DERMATOSIS IN WEANLING RATS FED LACTOSE DIETS

I. THE INFLUENCE OF RELATIVE HUMIDITY AND DIET [1]

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TWO FIGURES

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INTRODUCTION

In the course of studies concerning the nutritive value of goat's milk, cow's milk and purified lactose diets, a dry scaly dermatosis was observed on the paws of young albino rats (Collins, '50; Collins, Schreiber and Elvehjem, '51). The maximum degree of dermatosis appeared when weanling rats were fed the lactose rations for about 10 to 12 days, after which time the animals spontaneously recovered in an additional two or three weeks. Although weanling rats consistently developed dermatosis, young adult animals were observed to be more resistant to scale formation when placed on lactose diets.

A mild scaly paw condition has been reported by Geyer et al. ('43) to occur in rats fed a corn oil-lactose diet "when the humidity was not abnormally high." Further studies by Boutwell et al. ('44, '45) using this corn oil-lactose diet

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confirmed the earlier observation that this diet produced scaly dermatosis. This scale formation is apparently identical to the recently observed dermatosis resulting from a cow's or goat's milk diet.

Although the addition of folic acid and vitamin B_{12} to a mineralized goat's milk diet increased the growth rate of rats (Collins, Dietrich and Elvehjem, '50), these vitamins in addition to all other known water-soluble and fat-soluble vitamins plus ethyl linoleate gave no protection against the dermatosis (Collins, Schreiber and Elvehjem, '51).

The administration of fresh beef liver to weanling rats fed the vitamin B_{12}-low mineralized goat's milk diet resulted in an increased growth rate in accord with the vitamin B_{12} content of this supplement, and in addition provided definite protection against the dermatosis (Collins, '50). A similar anti-dermatosis activity of beef liver was observed in rats fed mineralized cow's milk or purified lactose diets (Collins, Schreiber and Elvehjem, '51; Schreiber, '51).

In view of these preliminary observations, experiments were conducted to study various aspects of this skin problem in greater detail.

EXPERIMENTAL AND RESULTS

Male weanling albino rats 35 to 45 gm of the Sprague-Dawley strain were reared individually in raised screen-bottom cages in the air-conditioned animal room of our laboratory and in chambers at controlled humidities.

The diets were fed ad libitum and in most cases either mineralized raw cow's milk or a purified diet similar to that shown in table 1. The cow's milk was obtained from the University of Wisconsin Creamery and was fed each morning and evening to minimize souring. The milk diets were mineralized to supply 3.0 mg of iron, 0.15 mg of copper and 0.15 mg of manganese per 100 ml of milk using iron pyrophosphate, copper sulfate and manganese sulfate, respectively. All supplements were fed each morning in individual

cups, while all incorporated materials were mixed uniformly in the ration.

In all of the diets which did not contain 28% fat, adjustments were made so that the protein, vitamin and mineral constituents would be fed at the same level per calorie as the diet containing 28% fat.

TABLE 1

Composition of the purified diet

BASAL MIXTURE	
	%
Fat	28
Carbohydrate	44
Casein (alc.-extracted)	23.5
Salts IV [1]	4
DL-methionine	0.25
L-cystine	0.25

FAT-SOLUBLE VITAMINS PER 100 GM OF DIET	
	mg
A (β-Carotene)	0.56
D (Calciferol)	0.014
E (α-Tocopherol)	2.24
K (Menadione)	0.21

WATER-SOLUBLE VITAMINS PER 100 GM OF DIET	
	mg
Thiamine	0.4
Riboflavin	0.6
Pyridoxine	0.6
Pantothenate (Ca)	3.0
Choline	150.0
Niacin	1.0
Biotin	0.02
Inositol	40.0
p-Aminobenzoic acid	10.0
Folic acid (Folvite)	0.50
B_{12} (Cobione)	0.005

[1] Hegsted et al. ('41).

The animals were always free from dermatosis when they were received from the animal farm. They were observed daily for skin irregularities and weighed weekly. To facilitate recording the extent of dermatosis, a numerical rating was arbitrarily established.

Numerical rating	*Degree of dry scaly dermatosis*
0	None (normal)
1	Slight
2	Moderate
3	Severe

A series of 4 pictures (fig. 1) taken of the dorsal side of the hind paws of rats illustrates the various degrees of dry scaly dermatosis.

The following aspects of this problem have been studied: (1) seasonal variation in the degree of scaly dermatosis and its relationship to relative humidity, (2) the effect of diet composition on dermatosis, and (3) the prevention of dermatosis by beef liver and other substances.

Seasonal variation in the degree of scaly dermatosis and its relation to relative humidity

The decrease in the degree of scaly dermatosis in rats fed cow's milk during the summer of 1950 and 1951 (fig. 2) has been shown to be due to the higher relative humidity in our

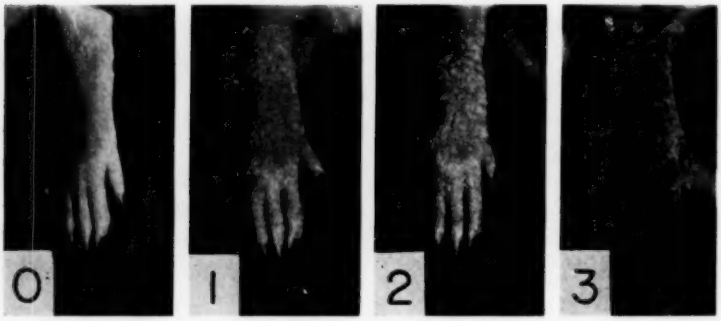

Fig. 1 A series of 4 pictures of the dorsal side of the hind paws of rats illustrating the various degrees of dry scaly dermatosis: 0 = None (normal); 1 = Slight; 2 = Moderate; and 3 = Severe.

animal room during this season. Experiments conducted at controlled low relative humidities in the chambers during the summer of 1951 demonstrated that summer milk produced a dermatosis similar to that of winter milk when the animals were maintained at comparable relative humidities. Our air-conditioned animal room is normally maintained at a relative humidity of about 30% during the winter and at 50 to 60% during the summer. The temperature is kept at approximately 26°C. throughout the year.

Low relative humidities were maintained in the chambers by controlling the inflow of dry air and by absorbing urinary

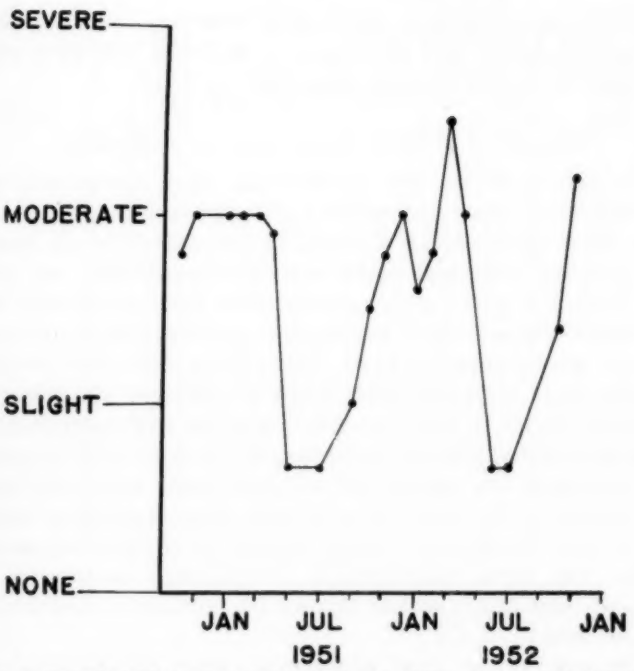

Fig. 2 The observed seasonal variation in the degree of dry scaly dermatosis in weanling rats fed a mineralized cow's milk diet.

and fecal water with anhydrous calcium chloride. The dry air was prepared by bubbling compressed air through a series of bottles containing concentrated sulfuric acid. The air was then passed through glass tubes filled with cotton and soda lime to remove acid vapors.

The minimum relative humidity which prevented the formation of the scaly dermatosis on the paws of animals fed lactose diets was a value between 50 and 60%.[3] As the relative humidity was decreased, the severity of scaliness increased from zero to three as shown in figure 1.

[3] Studies by Niño-Herrera, Schreiber, Collins and Elvehjem ('52) have shown that although scaliness does not occur at relative humidities above 50%, there is nevertheless histological evidence of skin irregularities.

A high humidity also accelerated recovery from the scaly dermatosis which had developed in milk-fed rats previously maintained at low relative humidity.

The effect of diet composition on dermatosis

The effect of the diet composition upon dermatosis was studied at a relative humidity of approximately 30% and at a temperature of 26°C., both in our animal room and in the humidity chambers under controlled conditions (see table 2). Rats fed cow's milk, goat's milk, corn oil-lactose, and coconut oil-lactose diets displayed a moderate degree of scaly dermatosis (groups one to 4). When butterfat-lactose or lard-lactose diets were fed only slight dermatosis was observed (groups 5 and 6). The substitution of an equimolar mixture of galactose and glucose for lactose in the corn oil diet (group 7) prevented the occurrence of dermatosis and accelerated the growth of the rats. Also, sucrose diets containing butterfat or corn oil (groups 8 and 9) did not precipitate any dermatosis. The more conventional low-fat diet containing 5% corn oil and 72% sucrose (group 10) similarly produced no dermatosis.

Changes in the level of corn oil from 20–28–35%, with corresponding reductions in lactose from 55–44–35%, had little effect in alleviating the moderate degree of dermatosis; however, the growth of the rats increased as the lactose level of the diet was decreased.

In accordance with the observations of Boutwell et al. ('45), the ceca of rats on diets containing lactose as the sole carbohydrate were larger and the contents were more fluid than the ceca from non-lactose-fed animals. Also weanling rats fed corn oil-lactose or coconut oil-lactose diets developed diarrhea which became apparent about two to 4 days after the animals were placed on experiment. Substitution of butterfat or lard for corn oil or coconut oil reduced the severity and duration of the diarrhea, whereas milk-fed animals showed little, if any, diarrhea. The presence of enlarged ceca with fluid contents and the varying degrees of diarrhea of lactose-fed

TABLE 2

The influence of diet upon dermatosis occurring on rats maintained at a relative humidity of approximately 30%

GROUP	DIET	NO. OF ANIMALS	AVE. WT. GAIN (TWO WKS.)	DERMATOSIS (TWO WKS.)	
				Incidence	Rating [2]
			gm		
1	Mineralized cow's milk	265 (22)[1]	52	260/265	2.0
2	Mineralized goat's milk	38 (7)	45	38/38	2.0
3	28% corn oil — 44% lactose [3]	123 (17)	55	121/123	2.0
4	28% coconut oil — 44% lactose	16 (3)	54	16/16	2.0
5	28% butterfat — 44% lactose	59 (6)	60	48/59	1.0
6	28% lard — 44% lactose	12 (2)	59	12/12	1.0
7	28% corn oil — 22% galactose + 22% glucose	17 (3)	73	12/17	0.5
8	28% butterfat — 44% sucrose	6 (1)	74	2/6	0.3
9	28% corn oil — 44% sucrose	73 (9)	72	65/73	0.5
10	5% corn oil — 72% sucrose [4]	17 (3)	62	11/17	0.5

[1] Figures in parentheses refer to the number of experiments.

[2] Numerical dermatosis ratings are based on the total number of animals on each diet.

[3] See table 1 for composition of this diet.

[4] This diet is isonitrogenous on a caloric basis with respect to the 28% fat rations with similar adjustments in the vitamins and the minerals.

animals could be contributory factors in what appears to be an initial dehydration of the skin of the paws followed by the occurrence of the dry scaly dermatosis.

Other abnormalities which were frequently observed in rats fed the corn oil-lactose and coconut oil-lactose diets were: (1) blood-like material coming from the nose, and (2) thinning of the hair on the abdomen of the animal. Following the recovery from diarrhea new hair was grown on the denuded areas.

The prevention of dermatosis by beef liver and other substances

As shown in table 3, the supplementation of ½ to 1 gm of fresh beef liver/rat/day (groups 5 and 15) was found to provide excellent protection from the scaly dermatosis and also to stimulate growth; however, the diarrhea occurring in corn oil-lactose-fed rats was not alleviated. Similar results were noted when fresh beef liver was supplemented to the rats on a corn oil-lactose diet at a level of 10% of the food consumed (group 14).

The effect of humidity on the anti-dermatosis activity of liver supplements has been studied under controlled conditions (in chambers) with animals fed a milk diet. At the relative humidity range of 15–25% the protective action of liver was very slight, while at the 25–45% range liver provided definite protection against scaliness. Above 50% relative humidity the scaly dermatosis did not occur (see part I), therefore, liver could not be evaluated for its scale-preventative action under these conditions. It is known, however (Niño-Herrera et al., '52), that histological changes did occur in non-supplemented milk-fed rats at relative humidities below and above 50%.[4]

The activity of beef liver was not destroyed when aqueous homogenates were autoclaved for one hour at pH 3, 7 and 10.

Preliminary work involving the fractionation of fresh beef liver using 60% methanol provided a partially active extract

[4] See footnote 2, page 129.

TABLE 3

Substances assayed for anti-dermatosis activity on mineralized cow's milk and 28% corn oil-44% lactose diets at approximately 30% relative humidity

BASAL DIET	GROUP NO.	MATERIAL ASSAYED	HOW ADMINISTERED	NO. OF ANIMALS	AVE. WT. GAIN (TWO WKS.)	DERMATOSIS (TWO WKS.)	
						Incidence	Rating [2]
					gm.		
I Mineralized cow's milk	1	.....	....	265 (22) [1]	52	260/265	2.0
	2	Non-essential amino acids [3] (195 mg/day)	Added to milk	12 (2)	61	10/12	0.9
	3	Beef muscle shanks (1 gm/day)	Supplemented	4 (1)	62	4/4	0.7
	4	Rat liver (1 gm/day)	Supplemented	18 (1)	62	6/18	0.4
	5	Beef liver (0.5–1 gm/day)	Supplemented	69 (10)	66	35/69	0.3
	6	.....	....	123 (17)	55	121/123	2.0
II Corn oil-lactose	7	Beef liver residue (5%)	Incorporated	12 (2)	63	12/12	1.8
	8	Beef liver (10%)	Incorporated	6 (1)	65	6/6	1.5
	9	Pork liver powder [4] (3%)	Incorporated	6 (1)	60	6/6	1.4
	10	Pork liver (1 gm/day)	Supplemented	6 (1)	65	6/6	0.7
	11	Beef kidney (1 gm/day)	Supplemented	6 (1)	59	6/6	0.5
	12	Beef muscle shanks (1 gm/day)	Supplemented	6 (1)	64	6/6	0.5
	13	Beef liver residue (5%)	Supplemented	12 (2)	63	10/12	0.5
	14	Beef liver (10%)	Supplemented	6 (1)	67	3/6	0.3
	15	Beef liver (1 gm/day)	Supplemented	59 (11)	66	31/59	0.3

[1] Figures in parentheses refer to the number of experiments.
[2] Numerical ratings are based on the total number of animals on each diet.
[3] L-tyrosine, L-cystine, DL-serine, L-glutamic acid, DL-aspartic acid, glycine and DL-alanine were fed in a proportion roughly approximating the levels found in beef liver.
[4] Dehydrated and defatted.

and an active residue. The residue retained its anti-dermatosis activity after ether extraction.

Liver residue and fresh liver were observed to be less active in preventing scaly dermatosis in rats when incorporated into corn oil-lactose diets at 5% and 10% levels, respectively (groups 7 and 8), than when supplemented at comparable levels (groups 13 and 14). Similar results were obtained using pork liver (see groups 9 and 10).

On a number of occasions active beef liver supplements were discontinued after two weeks of administration. The lack of storage by the rat of this anti-dermatosis activity was revealed when the moist, cool, smooth paws of liver-supplemented animals became dry, warm and rough within two to three days. This transition was followed by the development of a dry scaly dermatosis and a normal spontaneous recovery.

In addition to its role as a dermatosis-preventative agent, liver also accelerated recovery in initially non-supplemented lactose-fed animals.

Liver taken from rats which had developed the lactose-induced skin disorder possessed anti-dermatosis activity when supplemented to milk-fed animals as shown in table 3, group 4.

Those substances which were assayed by the method of supplementation to milk or corn oil-lactose diets and found to possess complete or nearly complete anti-dermatosis activity are presented in table 3. A number of other materials including animal and plant proteins were also assayed as incorporated constituents of the corn oil-lactose diet and found to have little or no protective action. However, inasmuch as the two most effective substances, fresh beef liver and liver residue, lost most of their anti-dermatosis activity when blended into the corn oil-lactose diet, all materials which were assayed by the incorporation technique must be re-assayed by the method of supplementation so that a comparable evaluation of their anti-dermatosis activity can be made with that of the beef liver supplement.

DISCUSSION

The seasonal variation in the degree of scaly dermatosis occurring in weanling rats fed milk diets (fig. 2) has been established to be the result of changes in relative humidity rather than nutritional differences in summer and winter milk. Although relative humidities above 50% have prevented the formation of visible scales, histological changes (Niño-Herrera et al., '52) were not prevented by high humidity.

As in the case of dermatoses resulting from various nutritional deficiencies, scaliness became more severe as the humidity was lowered. A situation analogous to that of the authors has been reported by Brown and Burr ('36) and Burr ('42) in which the scaly dermatosis resulting from an essential fatty acid deficiency was found to vary with relative humidity.

The purified butterfat-lactose diet produced a less severe scaly dermatosis than the natural butterfat-lactose diet (table 2, groups 5 and 1). This discrepancy may be the result of their different physical states. A dry whole milk powder diet has given variable degrees of dermatosis.

From tables 2 and 3 it is seen that the growth of animals on the non-lactose diets and the animals on liver-supplemented lactose diets was superior to that of the lactose-fed rats. From these comparisons one might surmise that a superior growth rate was instrumental in preventing the occurrence of scaly dermatosis. However, animals growing at different rates on the lactose rations exhibited the same degree of scaliness, although the recovery was more rapid among the faster growing animals. Furthermore, animals which grew at different rates on the non-lactose diets and on the liver-supplemented lactose diets did not develop dermatosis.

Beef liver supplementation to the rats fed lactose diets appeared to exert two separate effects, an anti-dermatosis action and a growth stimulation (table 3). When fresh liver or liver residue was incorporated into the corn oil-lactose

diet, the animals were not protected from the dermatosis, yet an increase in growth was noticed. Ershoff ('49) has reported a similar beneficial effect of liver upon the growth of weanling rats fed high lactose diets. The liver-supplemented rats would eat the liver within several minutes following administration, while animals receiving a diet containing incorporated liver necessarily ingested the liver at a slower rate. The rate of ingestion may, therefore, influence the anti-dermatosis action of liver.

SUMMARY

1. A dry scaly dermatosis was observed on the paws of weanling albino rats fed a mineralized milk or purified lactose diets. The maximum degree of scaliness occurred in 10 to 12 days followed by a spontaneous recovery in an additional two to three weeks.

2. The seasonal variation in the degree of dermatosis has been shown to be the result of seasonal changes in the relative humidity of our animal room rather than differences in summer and winter milks. Relative humidities above 50% prevented scaliness; decreasing values increased the severity of scaliness.

3. The purified lactose diets containing corn oil or coconut oil induced the most pronounced scaly dermatosis and diarrhea; replacement of these plant fats with butterfat or lard alleviated the dermatosis and diarrhea. Substitution of sucrose or a galactose-glucose mixture for lactose in the corn oil diet prevented the occurrence of dermatosis and diarrhea.

4. The supplementation of ½ to 1 gm of fresh beef liver per rat per day provided definite protection against the lactose-induced (milk-induced) dry scaly dermatosis when administered within a relative humidity range of 25 to 45%. Liver was more effective as an anti-dermatosis agent when supplemented than when incorporated in the purified lactose diets.

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THE EFFECT OF PEPTONE AND AMINO ACID INGESTION UPON THE CONCENTRATIONS OF TISSUE AMINO ACIDS[1]

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TWO FIGURES

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Mammalian tissues have been shown to contain significant quantities of free amino acids (Solomon et al., '51; Schurr et al., '50; Wiss and Kreuger, '49) involved presumably in the syntheses and breakdown of protein as well as in innumerable metabolic reactions. Wiss and Kreuger (Wiss and Kreuger, '49; Kreuger and Wiss, '49; Wiss, '49) recently studied the effects of high protein, high fat, or high carbohydrate diets upon individual free amino acids of liver, and Elvehjem and co-workers (Thompson et al., '50; Williams et al., '50; Denton et al., '50) measured individual tissue free amino acids following fasting, nitrogen deprivation, chilling, exercise, and amino acid deficiency. It is the purpose of the present paper to report the immediate changes in free and polypeptide amino acid concentrations in rat tissues following the ingestion of peptone. In addition, the tissues and blood plasma of rats were analyzed for individual free amino acids after the animals had been maintained upon diets supplemented with individual amino acids.

[1] This work was supported by grants from the Nutrition Foundation, Inc., and the University Research Board of the University of Illinois.

[2] Fellow of the National Institute of Health, U. S. Public Health Service; present address: Biological Research Laboratory, Southern Illinois University, Carbondale, Illinois.

The results indicate that the total concentrations of free amino acids in the various tissues, as well as the concentrations of the individual amino acids within a single tissue, undergo independent changes in response to the ingestion of protein or amino acids.

METHODS

Ten individual amino acids were measured by microbiological procedures previously described (Hier and Bergeim, '45; Sheffner et al., '48) except that acid produced by the growth of the organisms was measured by means of a quinhydrone electrode and the Cannon titration apparatus. The tissues were prepared for the measurement of free and polypeptide amino acids [3] by the method of Solomon et al. ('51). Briefly, this consisted of immediately freezing the excised tissues with solid CO_2, pulverizing them in a mortar, and extracting the powder with boiling dilute acetic acid in a Waring Blendor. The free amino acids in the tissue extracts and in plasma were determined by analysis of their tungstic acid filtrates.

EXPERIMENTAL

The immediate effects of peptone ingestion upon free and polypeptide bound amino acid levels of tissues

Adult male Sprague-Dawley rats weighing 250 to 400 gm were maintained on a Purina Fox Chow diet and fasted 24 hours prior to the study. Two independent experiments were performed. Four rats served as controls in each experiment. The remaining rats were fed 10 gm of Difco Bactopeptone per kilogram body weight by stomach tube. The rats were successively sacrificed in pairs one-half, one, one and one-half, two, 4 and 6 hours after being fed, and their tissues analyzed for free and non-protein bound amino acids.

[3] Polypeptide amino acids refer to the amino acids which become microbiologically available following hydrolysis of the tungstic acid filtrate and does not include the "free" form of the amino acids which is microbiologically available before hydrolysis.

Since the data from the two experiments were similar the results were averaged. The changes in concentration of free amino acids within the 4 tissues are presented in figures 1 and 2. In muscle, the values for arginine and lysine, which were present in high concentration in the peptone, increased greatly and those for the other amino acids showed no significant changes. The changes in levels in liver were for the

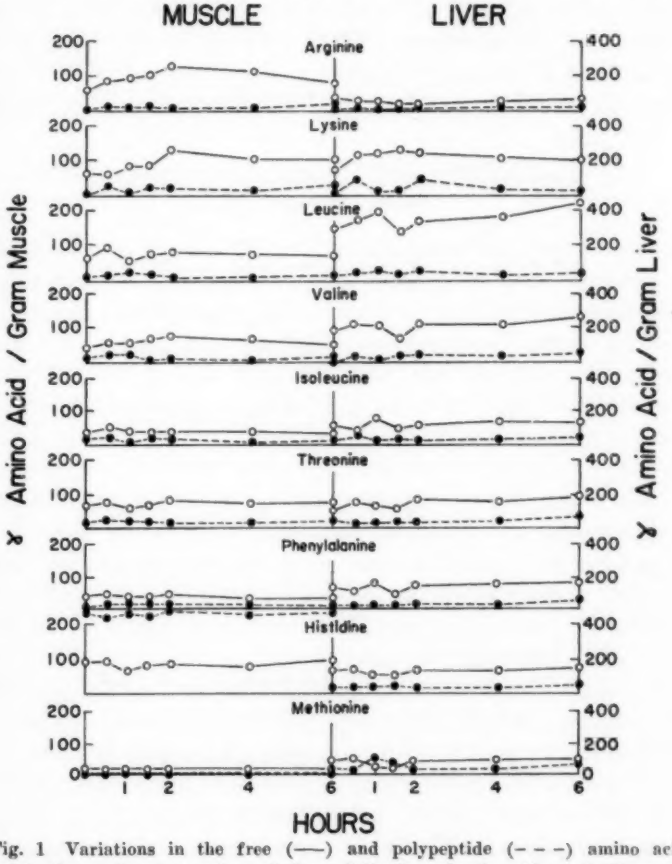

Fig. 1 Variations in the free (——) and polypeptide (– – –) amino acid concentrations of rat muscle and liver following the ingestion of 10 gm of peptone per kilogram body weight.

most part small, only the concentration of lysine increasing significantly; the values for lysine remained high for a prolonged period. In kidney, the free amino acid levels, other than that of leucine, rose in proportion to the quantities of each amino acid administered. Leucine increased more than the others in proportion to the amounts ingested. In general,

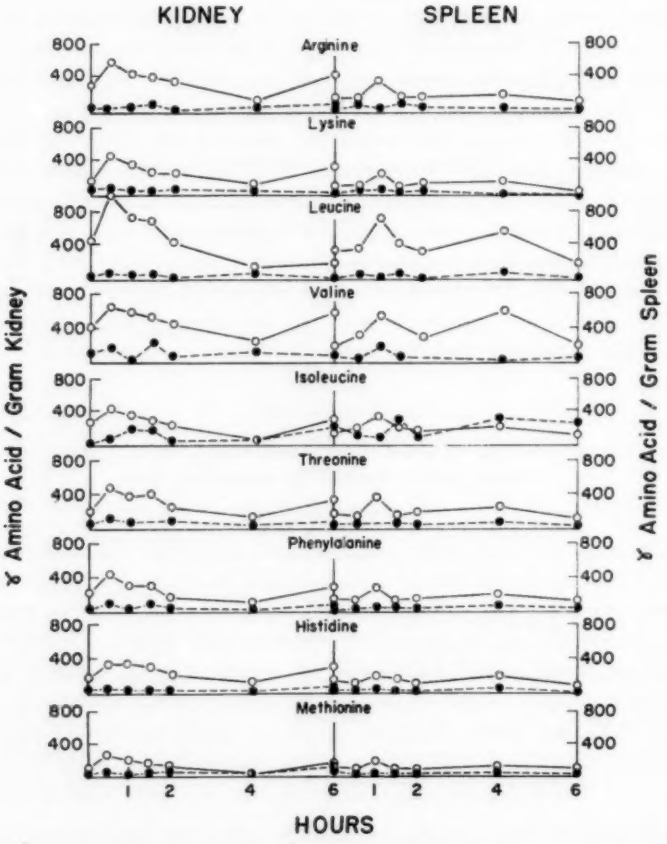

Fig. 2 Variations in the free (——) and polypeptide (– – –) amino acid concentrations of rat kidney and spleen following the ingestion of 10 gm of peptone per kilogram body weight.

kidney amino acid values rose in the first half hour and then fell gradually to below control values at approximately the 4th hour. There was also an immediate rise in spleen which tended to be related to the quantities ingested. The tissue amino acid levels for the two groups agreed within $\pm 15\%$.

The data obtained for the non-protein bound amino acids are also presented in figures 1 and 2. In general, values were low as compared to those for the free amino acids. The variations which resulted as a consequence of the ingestion of protein were also of a minor nature and were not significant.

Effect of supplementing the diet with methionine or phenylalanine

Adult white Sprague-Dawley rats were divided into two groups of 4 each. The first group received a basal diet while the second group received, in addition, a supplement of DL-methionine which increased the methionine content of the diet from 0.3% to 3%. The basal diet had the following composition: vitamin-free casein 10%, dextrose 25%, corn starch 50.2%, peanut oil 11%, salt mixture[4] 3%, L-cystine 0.3%; the vitamins included per kilogram were vitamin A 10,000 units, vitamin D 1,000 units, alpha tocopherol 200 mg, menadione 0.3 mg, riboflavin 8 mg, thiamine 8 mg, calcium pantothenate 16 mg, pyridoxine 8 mg, niacin 25 mg, biotin 0.3 mg, P-aminobenzoic acid 300 mg, inositol 2,000 mg, choline hydrochloride 2,000 mg, and folic acid 3 mg. In order to avoid unequal protein and caloric intake, the animals were fed individually at a level which coincided with the maximum intake of the animals receiving the methionine-supplemented diet. After 14 days on the described diets the rats were fasted overnight, sacrificed, and their kidney, liver, and muscle tissue and blood plasma analyzed for three amino acids, arginine, phenylalanine, and methionine. The results are pre-

[4] Hubbell, Mendell, and Wakeman salt mixture ('37).

sented in table 1. Significant [5] decreases in these animo acids occurred in kidney tissue. In liver, arginine rose, phenylalanine did not change appreciably, and methionine declined in concentration. In muscle and plasma, methionine increased considerably, while arginine and phenylalanine remained essentially the same.

TABLE 1

The effect of supplementary DL-*methionine in the diet upon the amino acid levels of rat tissues and plasma*

AMINO ACID	KIDNEY		LIVER	
	Control	Supplemented	Control	Supplemented
	μg/gm		*μg/gm*	
Arginine	153 ± 9.1 [1]	76 ± 7.7	19 ± 1.0	29 ± 2.2
Phenylalanine	116 ± 6.9	75 ± 5.4	71 ± 3.2	67 ± 3.9
Methionine	4.1 ± 0.9	0.8 ± 0.3	4.5 ± 0.7	1.3 ± 0.3
AMINO ACID	MUSCLE		PLASMA	
	Control	Supplemented	Control	Supplemented
	μg/gm		*μg/ml*	
Arginine	66 ± 5.1	57 ± 5.3	24 ± 2.1	20 ± 3.0
Phenylalanine	14 ± 1.5	15 ± 1.4	7.1 ± 0.5	8 ± 0.5
Methionine	0.5 ± 0.2	9.3 ± 3.5	3.2 ± 0.1	9.2 ± 1.5

[1] Standard error of the mean.

A second series of experiments was performed similar except that the basal diet contained 15% casein and the experimental diet contained in addition DL-phenylalanine to increase the phenylalanine content of the diet from 0.77% to 3.85%. The results of these experiments are presented in table 2. Contrary to what occurred on the high-methionine diet, on the phenylalanine-supplemented diet the fasting kidney arginine and phenylalanine levels rose significantly. The rise in kidney methionine was not statistically significant.

[5] Tests of significance were calculated from the formulae (Dixon and Massey, '51):

$$t = \frac{\bar{X}_1 - \bar{X}_2}{S_p \sqrt{\frac{1}{N_1} + \frac{1}{N_2}}}; \quad S_p^2 = \frac{N_1 S_1 + N_2 S_2}{N_1 + N_2 - 2}.$$

Also, in contrast to what occurred on the methionine supplemented diet, liver arginine decreased appreciably. In muscle tissue, decreases in arginine and methionine occurred on the high phenylalanine diet, but no changes were noted in the phenylalanine concentration. The amino acid levels in plasma remained essentially constant.

TABLE 2

The effect of supplementary DL-*phenylalanine in the diet upon the amino acid levels of rat tissues and plasma*[1]

AMINO ACID	KIDNEY		LIVER	
	Control	Supplemented	Control	Supplemented
	μg/gm		*μg/ml*	
Arginine	103 ± 5.8[2]	130 ± 8.7	34 ± 2.3	24 ± 1.4
Phenylalanine	74 ± 2.9	113 ± 7.7	54 ± 2.7	48 ± 2.5
Methionine	1.8 ± 0.5	2.9 ± 0.6	7.8 ± 0.7	6.2 ± 0.7
AMINO ACID	MUSCLE		PLASMA	
	Control	Supplemented	Control	Supplemented
	μg/gm		*μg/gm*	
Arginine	67 ± 2.0	52 ± 2.9	27 ± 1.0	27 ± 1.4
Phenylalanine	13 ± 0.6	13 ± 0.7	8.3 ± 0.6	8.0 ± 0.6
Methionine	5.9 ± 0.4	3.2 ± 0.3	5.6 ± 0.5	5.2 ± 0.3

[1] Each mean value represents the analyses from 4 rats.
[2] Standard error of the mean.

DISCUSSION

The response of the individual free amino acid levels in muscle to the ingestion of peptone corresponded to the quantity of the respective amino acids ingested. In liver, the changes were for the most part small. The prolonged high level of lysine probably reflects the slower rate at which lysine is metabolized in liver, whereas the decrease in arginine values indicate the increased arginase activity which occurs postabsorptively and which is associated with urea formation. In kidney, the free amino acid levels, other than for leucine, rose in proportion to the quantities of each amino acid administered; leucine increased more than the others in proportion to the amounts ingested. The relatively high levels of leucine in all the tissues after the ingestion of food

indicated that this amino acid is not utilized as rapidly as the others studied; in particular, the prolonged high concentrations in liver suggest that it is not destroyed as rapidly as the other amino acids.

An interesting observation made during this study was the fact that the large postprandial variations in free amino acids in kidney and spleen were accompanied by similar changes in the quantity of non-heat coagulable water soluble proteins which could be extracted from these tissues at pH 6. It could not be ascertained from the data whether or not the occurrence of larger quantities of these proteins was due to increased synthesis during the postprandial period of high-free amino acid levels.

The administration of a diet containing a high concentration of methionine resulted in decreased fasting kidney amino acid levels, including methionine. It is possible that amino acid catabolyzing enzymes were stimulated or increased in quantity. Data presented elsewhere (Sheffner and Bergeim, to be published) indicated that kidney L-amino acid oxidase activity is increased on a high methionine diet. However, since kidney L-amino acid oxidase is relatively low, it is likely that a general stimulation of catabolizing enzymes was responsible for the observed decrease in amino acid levels.

In the course of this work it was also noted that the free amino acid levels of leucine, valine, and threonine were significantly higher in muscle tissue of rats maintained upon a whole milk and bread diet than in the muscle of rats fed a Purina Chow diet containing considerably more of these amino acids.

The results therefore indicate that the concentration of free amino acids in tissues frequently undergoes changes in response to the ingestion of a protein, or amino acids which are unrelated to the amount of the particular amino acid ingested, and that single amino acids or various combinations of amino acids in the diet can influence the rate at which amino acids are utilized by the tissues.

SUMMARY

1. At various intervals of time following the ingestion of relatively large quantities of peptone, determinations were made in rats of the concentrations of 9 individual free amino acids in femoral muscle, liver, kidney, and spleen. In addition, measurements were made of variations in the individual non-protein bound amino acids of these tissues. The results indicate that the concentrations of individual amino acids within a single tissue undergo independent changes in response to the ingestion of peptone.

2. Supplementation of normal diets with large quantities of DL-methionine resulted in increased concentrations of methionine in plasma and muscle, but lower levels in kidney and liver tissue. Arginine and phenylalanine levels also decreased in kidney. On a diet containing high concentrations of DL-phenylalanine, no significant changes occurred in muscle and liver tissue and in plasma; contrary to what occurred on the high methionine diet, the levels of arginine and phenylalanine rose in the kidneys of fasted rats; kidney methionine concentrations did not change appreciably.

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THE EFFECT OF HEAT ON THE LYSINE AND METHIONINE IN SUNFLOWER SEED OIL MEAL[1, 2]

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The principal deficiency in commercial sunflower seed oil meal when used as a supplementary protein source in practical type diets for chicks and poults appears to be lysine (McGinnis, Hsu and Carver, '48; Slinger, Hill, Gartley and Branion, '49). The latter workers used an expeller-processed sunflower seed oil meal which analyzed 2.7% lysine on a 16% nitrogen basis. This value may be compared with 3.8% found by Block and Bolling ('45) in a solvent-processed meal. The evident discrepancy could be attributed to lysine destruction caused by exposure of the meal to high temperatures during the expeller processing, and is not unexpected in view of the conclusion of Clandinin and Robblee ('50) that the great variability in the nutritive value of commercial sunflower seed meals results from excessive processing temperatures.

The experiments described, herein, were conducted to study the effects of controlled conditions of heating on the lysine and methionine in sunflower seed oil meal. There is no evidence in the literature to suggest that methionine is deficient

[1] The data reported in this paper are taken in part from a thesis presented by J. C. Alexander in partial fulfillment of the requirements for the degree of Master of Science in Agriculture, University of Toronto, 1951. The investigation was supported in part by a grant from Toronto Elevators Ltd., Toronto, Ontario, and J. C. Alexander was holder of a fellowship from the Ontario Research Council.

[2] The authors are indebted to Co-op Vegetable Oils Ltd., Altona, Manitoba, for supplying the sunflower seed materials.

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in commercial sunflower seed oil meal from a nutritional standpoint. However, studies on this amino acid were included in view of the many published reports dealing with the effects of heat on its availability in other proteins.

MICROBIOLOGICAL STUDIES

Experimental

Cleaned dehulled advance Hybrid sunflower seeds [4] were coarsely ground, extracted for 24 hours in a soxhlet apparatus with diethyl ether and then finely ground. In one series of experiments the meal was heat-treated after extraction of the oil, and in a second series it was heat-treated before extraction.

Following the heat treatment the material was subjected to both acid and enzymatic hydrolysis. Acid hydrolysis was accomplished by autoclaving at 15 lb. pressure for 8 hours with 10% hydrochloric acid. Enzymatic hydrolysis was accomplished with pancreatin, by a procedure similar to that described by Melnick and Oser ('49) except that a borate buffer was used rather than a phosphate buffer. The enzymatic digestions were carried out for 120 hours at which time essentially maximum release of amino nitrogen and methionine had taken place.

Microbiological assays for lysine and methionine were conducted on the acid hydrolysates and for methionine on the enzymatic hydrolysates. Leuconostoc mesenteroides P-60 was employed for lysine and streptococcus faecalis no. 9790 for methionine. The medium and procedure was that of Henderson and Snell ('48) with some modifications. Tween 80 [5] was added to the medium at levels of 0.02% to 0.1% and assays were conducted in a total volume of 4 ml per assay tube. Amino nitrogen was measured as described by Melnick and Oser ('49).

[4] Previous to dehulling, the seeds had been passed through a Morris drier which dried them to a moisture content of about 7%.

[5] Polyoxyethylene sorbitan monooleate. Purchased from the Atlas Powder Co. Ltd., Brantford, Ontario.

When heat treatments were applied following extraction, the samples were heated in the containers used for the hydrolysis and these heated portions then hydrolyzed in toto. For acid hydrolysis 0.1 gm lots in pyrex test tubes were used, and for enzymatic hydrolysis, 6 gm in 125 ml erlenmeyer flasks.

When heat treatments were applied before extraction, the ground material was heated on metal trays in layers not exceeding one-half inch. There was a small variation in the protein content of the heated samples in this series. Accordingly, for comparative purposes all analytical values reported are based on the protein content ($N \times 6.25$) of the unheated meal.

Results and Discussion

The data in table 1 show that autoclaving and dry heating caused considerable destruction of lysine. The amount of destruction is roughly proportional to the duration of heating. It is also apparent that the presence of the oil in the meal during the heat treatment afforded some protection against lysine destruction during autoclaving for 15 and 30 minutes and during dry heating. The amount of destruction of methionine is negligible for all treatments. Some destruction is evident during heating before extraction of the oil, but even in this case the destruction found did not exceed 10%.

The susceptibility of lysine to moist heat destruction and the relative stability of methionine is in accord with similar investigations on soybean oil meal by Riesen, Clandinin, Elvehjem and Cravens ('47); Clandinin, Cravens, Elvehjem and Halpin ('47) and Evans and McGinnis ('48). However, it has been shown by Evans and Butts ('48) that dry heating of soybean oil meal is considerably less destructive to the lysine in the meal than is moist heating. In this respect, at least, soybean oil meal and sunflower seed oil meal respond differently to heat treatment.

Table 2 summarizes the findings on the release of methionine from sunflower seeds by enzymatic digestion. The striking thing about the data in table 2 is that for every sample

the percentage of the total methionine released exceeded the percentage hydrolysis of the protein. This would suggest that the methionine is being released in a form which is more active for streptococcus faecalis than is the free amino acid.

TABLE 1

Effect of heat on the destruction of lysine and methionine in sunflower seeds

HEAT TREATMENT [1]	TREATED AFTER ETHER EXTRACTION				TREATED BEFORE ETHER EXTRACTION			
	Lysine		Methionine		Lysine		Methionine	
	Content	Destroyed [2]	Content	Destroyed [2]	Content	Destroyed [2]	Content	Destroyed [2]
	%	%	%	%	%	%	%	%
None	1.80		0.96		1.80		0.96	
Autoclaving 15 minutes	1.56	13	0.96	0	1.73	4	0.89	7
Autoclaving 30 minutes	1.48	18	0.97	0	1.80	0	0.90	6
Autoclaving 2.5 hours	1.51	16	1.00	0	1.48	18	0.87	9
Autoclaving 5 hours	1.25	30	0.96	0	1.13	37	0.87	9
Autoclaving 10 hours	0.66	63	0.92	4	0.84	53	0.93	3
Dry heating 2.5 hours	1.38	23	0.93	3	1.72	4	0.93	3
Dry heating 5 hours	1.07	40	0.95	1	1.30	28	0.90	6
Dry heating 10 hours	0.74	59	0.92	4	1.17	35	0.93	3

[1] Autoclaving at 15 lb. pressure and dry heating in a hot air oven at 121°C.

[2] Decreases in the lysine and methionine content are considered to represent destruction of the amino acids.

Pader, Melnick and Oser ('48) have found evidence that peptides of lysine may produce a growth stimulation of assay organisms greater than that produced by an equivalent quantity of free lysine. On the other hand, methionine may be released quite early during enzyme digestion as compared

with the other amino acids. However, in experiments not reported here the authors have found a similar lack of agreement for lysine. This would suggest rather strong limitations on the use of microbiological assays for examining the amino acid content of incompletely hydrolyzed enzymatic digests.

Subject to these limitations, however, there is no clear evidence of extensive alteration of the methionine by heat treatment into a form from which biologically active methionine was not liberated by *in vitro* enzyme hydrolysis. Only

TABLE 2

Release of methionine from sunflower seeds by enzymatic hydrolysis

HEAT TREATMENT	TREATED AFTER ETHER EXTRACTION			TREATED BEFORE ETHER EXTRACTION		
	Per cent hydrolysis [1]	Per cent methionine found [2]	Per cent of total methionine [3]	Per cent hydrolysis [1]	Per cent methionine found [2]	Per cent of total methionine [3]
None	47	0.71	74	48	0.68	72
Autoclaving 15 minutes				29	0.69	70
Autoclaving 30 minutes				34	0.72	80
Autoclaving 2.5 hours	46	0.91	91	37	0.72	83
Autoclaving 5 hours	44	0.74	77	41	0.70	80
Autoclaving 10 hours	33	0.52	56	37	0.58	62
Dry heating 2.5 hours	40			38	0.58	62
Dry heating 5 hours	40	0.66	61	40	0.66	73
Dry heating 10 hours	37			42	0.71	76

[1] Formol titratable nitrogen as a percentage of the amount released from the unheated meal by hydrolysis with 8 N H_2SO_4 for 24 hours.

[2] Following 120 hours pancreatic digestion.

[3] Total methionine is assumed to be that found following acid hydrolysis of a similarly treated meal.

with 10 hours of autoclaving and two and one-half hours' dry heating does it appear that such an alteration has taken place. By these treatments, the methionine released as per cent of the total has been reduced from 74 to 56% for 10 hours' autoclaving following extraction of the oil, from 74 to 62% for 10 hours' autoclaving previous to extraction, and from 74 to 62% by two and one-half hours' dry heating. With milder autoclaving treatment the quantity of methionine released was actually increased.

CHICK GROWTH STUDIES

Experimental

Three feeding experiments were conducted in which the method of experimentation was similar. Day-old chicks were fed a commercial chick starter diet for a period of either 11 days or 14 days. They were then divided into experimental groups of equal numbers in such a way that each group was of approximately the same weight. In this allocation procedure extremely large and extremely small birds were discarded. The test diets were assigned to these groups and the gain in weight for the succeeding two weeks was taken as the criterion of response. During both the preliminary and experimental feeding periods the chicks were housed in battery brooders in an air-conditioned animal laboratory.

The experimental diets used were of a practical type but proved adequate for revealing lysine deficiencies. They were compounded by supplementing a basal diet with variously treated meals. The basal diet was of the following percentage composition of the final diets: ground corn 25.5, ground oats 12.5, ground oat groats 6.5, ground barley 6, ground wheat 15, dehydrated alfalfa meal 3, iodized salt 0.60, calcium carbonate 1, tricalcium phosphate 1.5, fortified fish oil (2400 A, 400 D) 0.24, manganese sulphate tetrahydrate 0.02, choline chloride (25% concentrate) 0.19, vitamin B_{12} supplement[6] 0.005, and riboflavin 0.1 gm per 100 lb. of diet.

[6] APF-3 supplied by Merck and Co. Ltd., Montreal, Quebec.

For each diet the balance to make 100% was composed of the variously treated meals, lysine when added, and a small additional amount of corn not exceeding 1.9% in any case. The meals were added in amounts to provide the comparable protein contents given in table 3.

In experiment 1, tests were conducted on a commercial sunflower seed oil meal. This experiment was conducted primarily to test the value of the experimental method to satisfactorily detect a lysine deficiency. In experiments 2 and 3 the solvent process meal that was used was prepared by a low temperature hexane extraction during which the maximum temperature was 160°F.[7]

The commercial expeller meal[8] used in experiment 2 was prepared from the same seeds as the solvent meal.

The meals were spread in thin layers on metal trays and then heated. All diets were fed ad libitum.

Results and Discussion

Table 3 gives the results of the three experiments. Only the mean gains in weight over the two-week experimental period are given. However, marked differences in the mean gain among the groups on the various diets were evident even at the end of the first week.

It is clear that the commercial expeller-process sunflower seed oil meals used in these experiments were markedly improved by supplementation with lysine (experiment 1, diets 1 and 2; experiment 2, diets 8 and 9). It is also evident that considerable lysine in the solvent process meal was destroyed or otherwise rendered unavailable to the chick by one-hour and two-hour autoclaving (experiment 2, diets 1, 2, 4, 5, 6 and 7). The solvent-process meal autoclaved one hour with added lysine promoted even better growth than the unheated meal. This finding indicates that one-hour autoclaving did

[7] Prepared by the research laboratories of Canadian Breweries Ltd., Toronto, Ontario, through the courtesy of Dr. W. E. Parker.

[8] Prepared by Co-op Vegetable Oils Ltd., Altona, Manitoba.

TABLE 3

Effect of heating on the feeding value of sunflower seed oil meal for chicks

EXPERIMENT [1]	DIET NO.	SUPPLEMENT TO BASAL DIET	PROTEIN IN DIET (N × 6.25)	LYSINE [2] IN DIET	MEAN GAIN [3] FOR TWO WEEKS	FEED-GAIN RATIO
			%	%	gm	
1 [4]	1	Commercial meal (expeller process)	23.9	0.5	75	5.34
	2	As 1 plus 0.4% L-lysine HCl	23.2	0.83	108	3.93
	3	As 1 autoclaved 1 hour at 15 lb.	23.5	0.45	47	7.94
	4	As 3 plus 0.4% L-lysine HCl	23.8	0.78	120	3.13
2	1	Solvent process meal	20.3	0.69	132	2.64
	2	As 1 plus 0.4% L-lysine HCl	20.2	1.02	141	2.46
	3	As 1 heated 30 min. at 100°C.	19.7	0.66	129	3.17
	4	As 1 autoclaved 1 hour at 15 lb.	19.8	0.55	80	4.48
	5	As 4 plus 0.4% L-lysine HCl	20.2	0.88	149	2.50
	6	As 1 autoclaved 2 hours at 15 lb.	19.6	0.37	51	5.87
	7	As 6 plus 0.4% L-lysine HCl	20.5	0.70	129	2.56
	8	Commercial meal [4] (expeller process)	19.5	0.55	79	4.02
	9	As 8 plus 0.4% L-lysine HCl	20.3	0.88	147	2.63
	10	Commercial soybean oil meal (solvent)	20.3	0.91	149	3.07
3	1	Solvent process meal	19.7		109	2.6
	2	As 1 autoclaved 5 min.	19.4		101	4.27
	3	As 1 autoclaved 10 min.	19.2		99	3.15
	4	As 1 autoclaved 20 min.	20.6		102	3.05

[1] Each group in experiment 1 contained 17 ♀ chicks, in experiment 2, 18 ♀ chicks, and in experiment 3, 8 ♂ chicks and 8 ♀ chicks. Chicks in experiments 1 and 2 were New Hampshire Barred Rock cross and in experiment 3, White Leghorns.

[2] For diets with added lysine, the lysine contents shown are the sum of the amount found by analysis of the unsupplemented diet plus the added lysine.

[3] Eleventh to the 25th day in experiments 1 and 2 and 14th to 28th day in experiment 3.

[4] Basal contained 0.2% added DL-methionine.

not affect nutrients in the meal other than lysine to any practical degree for the type of diet used.

There is evidence from the data that rather severe heating of the meal may actually improve its nutritional value provided the resulting lysine destruction has been compensated for (experiment 1, diets 2 and 4; experiment 2, diets 5 and 9). This improvement is small but consistent in the two experiments. Since the diets in experiment 1 contained added methionine, it seems unlikely that this result could be attributed to an increase in the availability of methionine.

In view of the well established fact that mild heat treatment improves the nutritional value of soybean oil meal, several relatively mild heat treatments were applied to the solvent process sunflower seed oil meal. Specifically, Evans, McGinnis and St. John ('47) showed that heating soybean oil meal for 30 minutes at 100°C. improved its nutritional value. Similar treatment in experiment 2 (diets 1 and 3) and autoclaving for 5, 10 and 20 minutes in experiment 3 failed to reveal any increase in growth promoting value.

It is of interest to note that no feather abnormalities in shape or color were observed on any of the lysine-deficient diets. Fritz, Hooper, Halpin and Moore ('46), Slinger, Hill, Gartley and Branion ('49), and Gartley, Slinger and Hill ('50) have reported that a lysine deficiency in the diet of Broad-Breasted Bronze poults results in poor pigmented feathers. The cross used in experiments 1 and 2 has dark colored feathers, which would readily have revealed any repigmentation.

Microbiological assays of the various sunflower seed oil meals used in feeding experiments 2 and 3 showed that autoclaving the solvent-process meal 30 minutes at 100°C., one hour at 15 lb. pressure, and two hours at 15 lb. pressure destroyed 10%, 21% and 51% of the lysine, respectively, and that the commercial expeller process meal had 30% less lysine than the solvent-process meal. The heat destruction of lysine thus recorded is considerably greater than that found with the meal used in the microbiological studies. Table 1 shows

30% destruction after autoclaving 5 hours at 15 lb. pressure. The sunflower seeds used in these two studies were from different crops and also the procedure used in preparing the extracted meals differed in the two cases. It may also be of significance that the sunflower seed material used in feeding experiments 2 and 3 was taken from a crop which was late in maturing and therefore contained many immature seeds. In any event the results suggest that there are factors which markedly influence the amount of destruction which may result from a given heat treatment.

SUMMARY

Autoclaving at 15 lb. pressure and dry heating at 121°C. destroyed a large proportion of the lysine in sunflower seed oil meal. Neither type of heating had an appreciable effect on methionine.

The findings support the conclusion that destruction of lysine during processing is probably the chief cause of the poor nutritional value for chick diets of commercial expeller-process sunflower seed oil meal.

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THE EFFECT OF FAT LEVEL OF THE DIET ON GENERAL NUTRITION

IX. THE RELATIONSHIP OF RADIATION INJURY IN THE RAT TO THE FAT CONTENT OF THE DIET[1, 2]

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TWO FIGURES

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Within the last few years, evidence has accumulated demonstrating the importance of fat in the diet (Deuel and Greenberg, '50). Rats receiving a generous quantity of fat in the diet were more resistant to such forms of stress as exercise (Deuel et al., '47; Samuels et al., '48), fasting (Samuels et al., '48), or hyperthyroidism (Greenberg and Deuel, '50) than were animals on a fat-low regimen. The present studies were undertaken to determine whether susceptibility to another type of stress, namely irradiation injury, might also be influenced by diet.

A number of investigations have shown an increase in susceptibility to x-ray injury when deficient diets are administered. Thus, Johnson et al. ('46) reported that young rats exhibited a markedly increased susceptibility to irradiation injury when fed a vitamin-free diet over that exhibited when the animals were maintained on an adequate dietary regimen.

[1] This work was carried out under a contract between the University of Southern California and the Atomic Energy Commission (No. AT(11-1)-113).

[2] Contribution 310 of the Department of Biochemistry and Nutrition, University of Southern California.

A considerable degree of protection could be obtained by daily oral supplementation with the B vitamins, given the rats prior to the x-irradiation. Thiamine has been reported to combat the nausea produced by irradiation injury in man (Wallace, '41; Whitmore, '43). In fact, Whitmore suggested that thiamine is required for the metabolism of the products of nuclear disintegration or of other substances arising as a result of irradiation.

Many substances have been used in attempts to modify irradiation sickness. Pyridoxine does not appear to influence the onset, severity, or duration of the leukopenia following irradiation, but it does afford a definite protection against delayed effects of the Roentgen rays. Goldfeder et al. ('48) found that, if the leukopoietic system of their experimental animals returned to normal, they did not die of delayed reactions. In this respect, pyridoxine hydrochloride resembled folic acid. Both these substances decrease, and in some cases prevent, diarrhea in irradiated mice. They may also extend the survival time of the animals. On the other hand, most reports on folic acid have been negative. These studies concerned principally leukopenia in cats (Adams and Lawrence, '48), swine (Cronkite et al., '50), and rats (Jacobson et al., '48; Stearner and Jacobson, '47; Stearner, '48). Carter and co-workers ('50) have likewise demonstrated that crystalline vitamin B_{12} does not prolong the survival of rats exposed to x-rays.

Ershoff ('52) has reported that the survival time of immature and young female rats following multiple sublethal doses of x-irradiation was significantly prolonged on a liver-containing diet over that on a ration high in the known synthetic B vitamins. However, no significant differences were noted in survival time between the rats on the several dietary regimens when a single lethal dose of x-irradiation was employed.

Protein-depleted rats have been reported to be more susceptible to penetrating radiation than are animals on a diet containing adequate protein (Jennings, '50). This finding

is in line with the observation of Ross and Ely ('49) that the metabolism of tyrosine, tryptophan, and arginine is upset by x-irradiation.

Protection from x-irradiation injury has also been obtained by the use of specific compounds known to be important in the functioning of the epidermis. Thus, beneficial effects have been reported in a number of cases with rutin and other compounds resembling vitamin P and having a flavonoid structure. Griffith et al. ('47) and Sokoloff and co-workers ('50a, b) obtained positive results with rats, while Rekers and Field demonstrated increased survival in dogs subjected to low dosages of x-irradiation ('48; Field and Rekers, '49); however, the latter workers ('48) did not find that the flavonoids exerted any beneficial effects when high dosages of x-irradiation were used. Clark and collaborators ('48) have reported that a flavonoid prepared from lemon protected guinea pigs against x-irradiation damage. However, Cronkite and associates ('49, '51b) have consistently noted negative results in the case of mice treated with flavonoid compounds, as have also Kohn et al. ('48) working with rats. Haley and Mann ('52) have also recently reported negative results from the use of rutin in protecting guinea pigs from x-ray injury. There is no evidence that the positive results obtained with citrus products is to be attributed to ascorbic acid, since tests with this substance have yielded only negative results (Simmons et al., '46; Field and Rekers, '48, '49).

Cysteine and related sulfur derivatives are compounds of importance in relation to the epidermis which have likewise been shown to be active in protection from x-irradiation. Cysteine was found to be an effective protective agent against x-irradiation when given intravenously or orally to rats or mice (Patt et al., '49, '50a, b; Goldie et al., '51; Swift et al., '52). Patt, Smith and Jackson ('50a) found that intravenous injections of cysteine prior to irradiation reduced the mortality rate from 66 to 22%; they also observed that the leukopenia and erythropenia were less severe and of shorter duration in the pretreated rats. Similar results were also reported

by Smith et al. ('50). This amino acid appeared to be active when given at considerable periods prior to exposure to x-rays, but was ineffective when given shortly after irradiation. Apparently it is necessary for this substance to be in the blood and tissues at the time of exposure to the Roentgen rays in order to counteract their effects.

Some improvement in survival time, and a decrease in weight loss, in rats after x-irradiation, followed treatment with the cysteine derivative, glutathione (Chapman and Cronkite, '50; Chapman et al., '50), although the results on mice were essentially negative (Cronkite, Brecher and Chapman, '51a).

BAL has also been shown to have a protective action against x-irradiation in mice (Works et al., '50) and in rats (Hursh, '49). Another sulfur compound, thiourea, has likewise been found to possess some potency as a protective agent in preventing x-ray injury in mice (Limperos and Mosher, '50), although Haley et al., ('50) reported negative results in tests on a strain of mice which differed from that used by the former investigators. The recent reports of Selle ('49, '52), listing all the agents which have been used in an attempt to counteract the effects of ionizing radiations, also include a discussion of the effects of cyanides, nitrides, antibiotics, antihistaminics, adrenocortical extracts, sex hormones, splenic implants, and environmental factors.

Decker et al. ('50) reported typical symptoms of acute essential fatty acid deficiency produced by x-irradiation in fat-depleted mice. They also noted a greater survival of mice on a fat-free diet plus corn oil than on the control diet. The present experiments were carried out to investigate the effect of dietary fat on injury due to exposure to x-irradiation.

EXPERIMENTAL

Three series of tests were carried out on rats receiving diets varying in fat content, to determine the effect of a single large dose or of repeated sublethal doses of x-irradiation on

survival, changes in body weight, hemoglobin and white cell count.

In series I, a single dose of x-irradiation was given at levels of 650 or 850 *r* to young male and female rats (approximately 10 to 12 weeks of age) which had received a fat-free diet (diet 1) or a high-fat diet (diet 4) for three to 4 weeks prior to the tests. The higher level of irradiation was such that one would expect a mortality of 50% within 30 days after exposure to the rays. In series II, adult rats which had previously received a fat-free diet (diet 1), or diets containing large amounts of fat (diets 3 and 4), from weaning at three weeks of age for 15 months, were exposed to 300 *r* weekly for 4 weeks; later, from the 7th to the 13th weeks, they were exposed on alternate weeks.

The tests described as series III were carried out on young rats in a manner somewhat similar to that employed in series II. Upon weaning, the rats were placed on diets 1 and 4. After receiving this regimen for three weeks, they were redistributed into three groups which were fed diets 1, 2 (2% fat) and 4 for the rest of the test. After 6 weeks on the new diet, the animals (at the age of 12 weeks) were subjected to the first exposure of 300 *r*. Control groups which received no irradiation were included in series III.

The rats were of the U.S.C. strain. They were kept in individual cages and were weighed daily. Blood for hemoglobin determination and white cell count was obtained from the tail. In order to allow sufficient time for the tail to heal, and to lessen the probability of infection, samples were removed from the same rat only once in two or three weeks. Hemoglobin was determined by the use of the Haden-Hauser hemoglobinometer, and white cell counts were carried out by the usual procedure, using a Spencer Bright Line hemocytometer. The control leukocyte counts obtained before irradiation were the average figures obtained on all the rats of each group. The mean value of the controls was used for the control value in calculating the decrease in leukocyte

count after x-irradiation on the individual rats. Values for hemoglobin could not be determined below a value of 7.5%.

The composition of the experimental diets used is included in table 1. The control diet was a commercial laboratory ration,[3] which contains approximately 5% fat.

TABLE 1

Composition of diets

COMPONENTS [1]	DIET			
	1	2	3	4
	%	%	%	%
Casein, commercial	25.0	25.2	29.5	34.0
Sucrose	66.84	65.64	46.31	26.28
Cottonseed oil [2]	0	1.0	14.0	29.0
Cellulose [3]	3.0	3.0	4.0	4.0
Salt mixture [4]	4.0	4.0	5.0	5.5
Water-soluble vitamin mixture [5]	0.16	0.16	0.19	0.22
Fat-soluble vitamin mixture [6]	1.0 [7]	1.0	1.0	1.0

[1] The folic acid was kindly furnished by Lederle Laboratories and the biotin by the Hoffmann-LaRoche Co. The remaining B vitamins and α-tocopherol were given to us through the kindness of Merck and Co. Crystalline vitamin D_3 was generously furnished us by the Winthrop Chemical Co.

[2] Wesson oil.

[3] "Solka-floc" obtained from the Brown Company, San Francisco.

[4] Osborne-Mendel salt mixture (J. Biol. Chem., 1917, *32:* 309).

[5] The water-soluble vitamin mixture had the following composition: choline chloride, 91.5%; thiamine chloride hydrochloride, 1.24%; riboflavin, 1.24%; pyridoxine hydrochloride, 1.24%; calcium pantothenate, 2.48%; nicotinic acid, 1.83%; folic acid, 0.30%; and biotin, 0.01%. Vitamin B_{12} (dissolved in ethyl alcohol) was added to the extent of 0.01%. This also contains 0.15% menadione.

[6] The cottonseed oil solution of fat-soluble vitamins had the following composition per 100 gm: α-tocopherol, 500 mg; carotene (90% — β and 10% — α), 20 mg; crystalline vitamin D_3, 0.5 mg.

[7] Made up in propylene glycol instead of cottonseed oil.

The irradiation of the rats was carried out by the use of a General Electric Maximar 250-III 200/260 volt apparatus.[4] The radiation chamber was a wooden box, having a plastic top made of cellulose acetate sheets ⅛ inch in thickness, which was used to hold the rats during exposure to the x-rays. This

[3] Purina laboratory chow, Ralston Purina Co., St. Louis, Mo.

[4] For a more complete description of this apparatus, see Ershoff ('52).

was divided into a number of compartments, with cellulose acetate sheeting, which were large enough to allow the rats to remain in them without discomfort but too small to permit any change of position. The intensity of the irradiation was standardized by measurement with a Victoreen *r* meter within the compartments. The plastic cover was found to absorb only 1.5% of the irradiation. The calculation of the dosage is based upon the measurement made on the top of the radiation chamber. If the measurement is made on the floor of the chamber with the cover in place, the value is approximately 18% lower.

During the period in which the rats were undergoing x-ray treatment, the radiation chamber was constantly rotated at a slow speed (2 R.P.M.) on a rotatory table. This ensured an equal distribution of x-irradiation in all positions within the box. To prevent overheating during the x-ray treatment, a fan, directed toward the box, was operated whenever irradiation was in progress. Numerous tests were carried out which demonstrated a constancy in the radiation dosage emitted over a period of days by the x-ray tube.

The LD_{50} was determined for rats maintained on the laboratory stock diet. In the case of adult male and female rats, the LD_{50} was found to be 710 *r*, while in young adult rats a figure of 625 *r* was obtained.

RESULTS

Experiments on single exposure to radiation

Table 2 gives the mortality figures, as well as the values for the hemoglobin and leukocyte counts, for male and female rats previously on a fat-free diet or a 30% fat regimen, after having been subjected to 650 or 850 *r* of irradiation in a single dose (series I). The mortality rate averaged only 14% for the rats receiving the lower dosage of x-irradiation, while 53% of the rats receiving the higher dosage died. A total of 374 rats was used for the tests; these were approximately equally divided between the two dosage levels.

TABLE 2

The mortality, hemoglobin level, and white cell count of male and female rats for 46 days after irradiation with 650 or 850 r in a single dose (diet 1, 0% fat; diet 4, 30% fat)

Series I

DAYS AFTER IRRADIATION	MORTALITY, %				HEMOGLOBIN, % [a]				LEUKOCYTES, % STARTING LEVEL [b]			
	Males		Females		Males		Females		Males		Females	
	Diet 1	Diet 4	Diet 1	Diet 4	Diet 1	Diet 4	Diet 1	Diet 4	Diet 1	Diet 4	Diet 1	Diet 4
650 *r* (Rats per group: males, diet 1, 48; diet 4, 37; females, diet 1, 50; diet 4, 50).												
0	..	..	..	..	12.7	12.5	12.8	12.8	..	..	..	..
7	8	3	4	0	12.1	11.8	11.9	10.4	27	15	13	14
13	17	3	12	8	9.0[1]	10.6	14.7	8.7	38	30	26	31
19	23	3	14	14	11.2	9.7	11.5	11.3	32	77	35	73
25	23	8	14	14	12.4	11.7	13.0	12.3	35	60	50	83
31	23	8	14	14	13.4	14.0	13.3	13.9	84	73	61	84
37	23	8	14	14	13.8	12.7	13.3	12.8	55	92	76	72
43	23	8	14	14	15.3	12.9	12.9	13.1	65	89	107	91
46	23	8	14	14	13.8	13.5	12.7	13.5	54	84	120	105
850 *r* (Rats per group: males, diet 1, 49; diet 4, 40; females, diet 1, 50; diet 4, 50).												
0	..	..	..	..	12.7	12.5	12.8	12.8	..	..	..	..
7	4	10	2	8	11.8	12.1	12.1	12.0	7	12	7	10
13	36	43	46	28	7.6[3]	8.3[3]	7.6[5]	7.9[3]	12	11	29	19
19	45	55	54	42	9.0[1]	7.7[3]	8.7[1]	9.1[3]	46	33	59	39
25	49	55	56	46	12.3	8.9[3]	13.3	11.4	46	37	63	71
31	51	55	56	46	14.3	11.1[1]	13.3	12.2	41	45	70	69
37	51	55	56	46	14.8	12.3	13.7	12.6	48	66	76	63
43	51	59	56	46	13.9	12.8	14.4	13.2	46	68	90	90
46	51	59	56	46	14.0	12.8	13.6	12.4	53	73	89	69

[a] The superscript numbers indicate the number of rats having a hemoglobin level too low for accurate determination. These are calculated as 7.5%.

[b] Starting counts were as follows: males (diet 1), 21,500; males (diet 4), 19,000; females (diet 1), 17,200; females (diet 4), 17,500.

No appreciable differences in mortality rate can be attributed to the diet. Likewise, no differences in susceptibility to x-irradiation were found to be related to sex.

Little effect on blood hemoglobin values was noted by the 7th day after exposure to x-rays; following this period there was a rapid decline, which reached the minimum value between the 13th and the 19th day. There would appear to have been a somewhat greater resistance to a decrease in the hemoglobin level in the rats on the fat-free diet than in those animals on the diets containing fat. The average values of the hemoglobin for the 8 tests, made after exposure to 650 *r*, were 12.6 and 12.1%, respectively, for the male rats on diets 1 and 4, and 12.9 and 12.0%, respectively, for the females on these two diets. In the case of the tests on the higher dosage (850 *r*), the mean hemoglobin values were 12.2 and 10.8% for the males and 12.1 and 11.4% for the females on diets 1 and 4, respectively. These averages are of limited value since, with the method employed, there is no way to estimate the figures for hemoglobin below 7.5%.

In the case of the blood leukocytes, the minimum level was obtained in all cases in the first post-radiation sample; namely, at 7 days. In surviving animals the value slowly returns to normal, although the recovery is not complete at 46 days in the case of the males after 650 *r*, or in either sex after the 850 *r* dosage. The average percentage for the pre-irradiation leukocyte level found over the 46-day period following dosage with x-irradiation was higher in all cases for females than for males. In three of the 4 groups, the recovery based on white cell count would appear to have been slightly better in the case of the rats on the high-fat diet (diet 4). Thus, the mean percentages of the pre-irradiation leukocyte levels found after exposure to 650 *r* were 49 and 65 for the males, and 61 and 69 for the females receiving diets 1 and 4, respectively. In the tests carried out at the higher x-irradiation dosage the comparative figures are 37 and 43 for the males and 60 and 54 for the females on diets 1 and 4, respectively.

Experiments with repeated sublethal exposure to x-irradiation

Although there was no indication that diet plays a role in the protection of rats from a single exposure of x-irradiation at a fairly high level, it was believed that variations in response might be noted if the stress were less severe and were continued over a longer period. The method employed was to administer single sublethal doses of 300 *r* weekly over a 14-week period, omitting irradiation during 6 different weeks, so that a total of 2,400 *r* was given. In series II the rats received diets containing 15 or 30% of fat (diets 3 and 4), in addition to the fat-free regimen (diet 1), while in series III the fat levels chosen were 0, 2 (diet 2) and 30%. In all cases the fat employed was cottonseed oil, which is known to contain about 50% of essential fatty acids. Assuming a daily food consumption of 10 gm of diet, this would mean that approximately 100 mg of linoleate would be consumed per day by the rats receiving diet 2. The intake of linoleate would probably exceed 600 mg per day with diet 3 and 1,000 mg per day with diet 4. The mortality figures for these tests are recorded in tables 3 and 4.

In both series of tests the rats receiving the diets containing fat showed a marked superiority in resistance to x-irradiation compared with those on the fat-free regimen. In series II the beneficial effects were equally pronounced when the diets contained 15 and 30% of fat. No sex differences in susceptibility to x-irradiation, irrespective of diet, were noted in the tests recorded in table 3. The survival time for the males and females on the fat-free diet averaged 34 and 39 days, respectively, compared with values of 70 and 76 days for the rats on diet 3 (15% fat) and 63 and 69 days for the animals on diet 4 (30% fat). The differences in survival time between the control groups and the experimental groups were highly significant in all cases. The increased survival time occurred in spite of the fact that the average exposure to x-irradiation was 40 to 50% greater for the rats on the fat diets than for those on the fat-free regimen. This situa-

TABLE 3

The percentage of mortality of male and female rats on diets containing several levels of fat when subjected repeatedly to sub-lethal doses of x-irradiation (diet 1, 0% fat; diet 3, 15% fat; diet 4, 30% fat)

Series II

WEEKS AFTER IRRADIATION	TOTAL CUMULATIVE IRRADIATION	MALE RATS [1]			FEMALE RATS [1]		
		Diet 1 (18)	Diet 3 (24)	Diet 4 (20)	Diet 1 (17)	Diet 3 (20)	Diet 4 (20)
	r						
1	300	0	0	0	0	0	0
2	600	0	0	0	5.9	5.0	5.0
3	900	5.5	8.3	5.0	17.7	5.0	5.0
4	1,200	33.3	16.7	20.0	23.5	15.0	5.0
5	1,200	72.4	20.8	30.0	47.0	15.0	10.0
6	1,200	77.8	20.8	40.0	53.0	20.0	10.0
7	1,200	94.4	29.2	45.0	70.7	25.0	20.0
8	1,500	94.4	33.3	45.0	82.4	30.0	35.0
9	1,500	94.4	33.3	50.0	82.4	30.0	40.0
10	1,800	94.4	41.7	55.0	94.2	30.0	40.0
11	1,800	94.4	45.8	55.0	100.0	30.0	45.0
12	2,100	100.0	50.0	60.0	..	45.0	60.0
13	2,100	..	62.5	75.0	..	55.0	85.0
14	2,400	..	83.4	80.0	..	70.0	100.0
15	2,400	..	95.8	90.0	..	85.0	..
16	2,400	..	100.0	100.0	..	100.0	..
Average day of death [2]		34 ± 3.3	70 ± 6.1	63 ± 7.1	39 ± 4.3	76 ± 7.0	69 ± 5.3
			5.22	**3.67**		**4.52**	**4.42**
Average exposure per rat in r [2]		1,235 ± 54	1,860 ± 112	1,710 ± 119	1,270 ± 77	1,950 ± 128	1,800 ± 111
			5.08	**3.63**		**4.55**	**3.92**

[1] The figures in parentheses at the head of the columns represent the number of rats in each group.

[2] Including the standard error of the mean calculated by the formula, $\sqrt{Ed^2/n - 1/\sqrt{n}}$.

The boldface figures are the ratios of mean difference (experimental — control) and $\sqrt{(S.E.M.)^2_c + (S.E.M.)^2_e}$. A value in excess of 3.0 indicates significance.

TABLE 4

The percentage of mortality of male and female rats on diets containing several levels of fat when subjected repeatedly to sub-lethal doses of x-irradiation (diet 1, 0% fat; diet 2, 2% fat; diet 4, 30% fat)

Series III

WEEKS AFTER IRRADIATION	TOTAL CUMULATIVE IRRADIATION	MALE RATS [1]			FEMALE RATS [1]		
		Diet 1 (30)	Diet 2 (30)	Diet 4 (26)	Diet 1 (26)	Diet 2 (30)	Diet 4 (29)
	r						
1	300	0	0	0	0	0	0
2	600	0	3.3	0	3.8	0	3.4
3	900	3.3	3.3	0	7.7	0	6.9
4	1,200	10.0	3.3	3.8	11.5	3.3	10.3
5	1,200	20.0	10.0	7.7	11.5	3.3	10.3
6	1,200	33.3	10.0	11.5	11.5	3.3	13.8
7	1,500	43.3	13.3	11.5	15.4	10.0	13.8
8	1,800	56.7	23.3	19.2	23.1	23.3	20.7
9	2,100	73.3	36.7	23.1	53.8	40.0	51.7
10	2,400	90.0	63.3	57.7	73.0	53.3	68.9
11	2,400	93.3	76.7	73.2	84.6	70.0	86.2
12	2,400	100.0	90.0	80.8	88.5	80.0	96.5
Surviving at end		0.0	10.0	19.2	11.5	20.0	3.5
Average day of death [2]		51 ± 3.1	65 ± 3.0	67 ± 3.2	61 ± 3.6	67 ± 2.6	62± 3.4
			3.25	**3.60**		**1.35**	**0.20**
Average exposure per rat in r [3]		1,770 ± 99	2,205 ± 92	2,290 ± 82	1,980 ± 84	2,170 ± 62	2,080 ± 87
			3.22	**4.07**		**1.82**	**0.82**

[1] The figures in parentheses at the head of the columns represent the number of rats in each group.

[2] Including the standard error of the mean calculated by the formula, $\sqrt{Ed^2/n - 1/\sqrt{n}}$.

The boldface figures are the ratios of mean difference (experimental — control) and $\sqrt{(S.E.M.)^2_c + (S.E.M.)^2_e}$. A value in excess of 3.0 indicates significance.

tion obtained because irradiation was continued as the experiment progressed, and the rats which survived were obviously exposed to greater doses than had been those animals which succumbed earlier.

In general, the data presented for series III in table 4 confirm the results of series II. However, it is shown that cottonseed oil is almost equally as effective in affording protection from the toxicity of irradiation at a 2% level in the diet as at a 30% level. In contradistinction to the results obtained in series II, a marked sex difference in resistance to x-irradiation was noted on the several diets in series III. The males receiving either diet 2 or 4 had a considerably higher survival rate throughout the test. The average time of death was 51 days for the male rats on the fat-free diet (diet 2), 65 days for the animals on diet 2 (2% fat) and 67 days for those on diet 4 (30% fat). These variations are likewise highly significant.

On the other hand, the variation in the survival time of the female rats in the several groups was slight, the values being 61, 67, and 62 days, respectively. It is apparent that the failure to demonstrate any significant increase in survival time in the females on the fat-containing diets is due to the fact that the females in the control group lived for a longer period than did the animals in the control group which was composed of male rats. Actually, the average number of days of survival on the two fat diets was quite similar in the corresponding male and female groups.

There are several possible explanations for the variations found in series II and III. The animals in series II were approximately 18 months old, while those in series III were young animals less than three months old at the start of the tests. A second difference is the rate at which the x-irradiation was given to the animals. In series II, 14 weeks were required for the administration of 2,400 *r;* no x-irradiation was given between the 4th and the 7th week or during the 9th, 11th, and 13th weeks. In the case of series III, the total

x-irradiation covered a period of only 10 weeks; x-irradiation was omitted only from the 4th to the 6th week.

The data have also been subjected to statistical evaluation by the use of the probit scale or the N.E.D. (normal equivalent deviate) as described by Moore, Cramer and Knowles

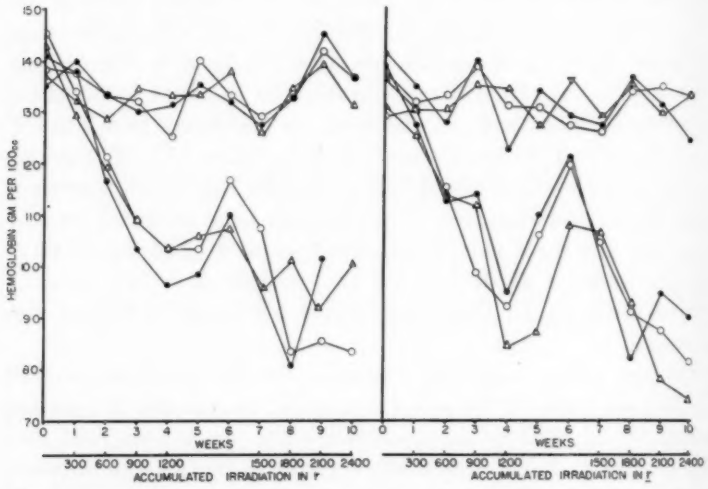

Fig. 1 Average values for hemoglobin in grams per 100 ml of blood of male rats (left) and female rats (right) before and after x-irradiation with 2,400 *r* over a 10-week period. The upper group of curves represents hemoglobin values for the normal controls, and the lower group of curves that for the irradiated animals. Curves with black circles represent data for animals on a fat-low diet (diet 1); those with open circles present data for rats on a 2% fat diet (diet 2); and those with open triangles give results for rats on a 30% fat diet (diet 4).

('51). On the basis of such an analysis [5] it was shown that frequency of death, calculated for the 42nd day after all groups had received 1,200 *r*, with the sexes combined, involved highly significant differences when the results for rats on the fat-free regimen are compared with those for animals on the fat-containing diets. The value for "p" was < 0.001 for

[5] The authors wish to thank Professor Fred Moore and Mr. Robert Knowles of the Department of Experimental Medicine of the School of Medicine of the University of Southern California for their aid in some of the statistical evaluations.

series II and $< 0.02, > 0.01$ for series III. That the age of the animals plays a role is likewise shown by the fact that a highly significant figure is obtained when data on the rats on the fat-free diets in series II and III are compared (<0.001) or when the frequencies of death of the rats receiving the

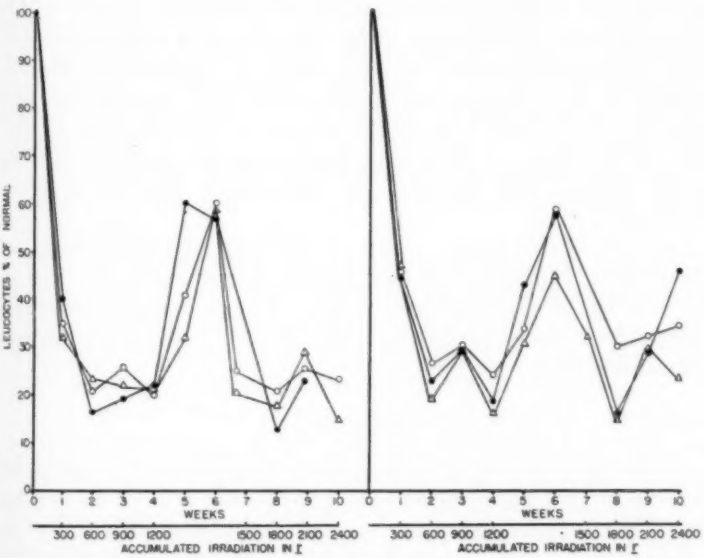

Fig. 2 Average values for leukocyte count in per cent of normal in male rats (left) and female rats (right) after irradiation with 2,400 r over a 10-week period. Curves with black circles represent data for animals on a fat-low diet (diet 1); those with open circles present data for rats on a 2% fat diet (diet 2); and those with open triangles give results for rats on a 30% fat diet (diet 4).

fat diets in the two series are compared ($p = < 0.01, > 0.001$). However, this method of statistical treatment fails to show clear differences between the sexes or between the responses to the several levels of fat.

Figure 1 summarizes the changes in hemoglobin, while figure 2 gives a summary of the variation in leukocyte count, of series III during the 10-week period of the experiment.

As would be expected, the hemoglobin level remained at a subnormal value during the entire experiment. However, in line with the lower dosages of x-irradiation employed, the rate of decrease in hemoglobin was less in the present tests than in either experiment in series I, in which a single exposure to a large dosage was used rather than repeated exposures to sublethal levels of x-irradiation, as occurred in series III. The minimum hemoglobin value was reached during the 4th week and again during the 10th week (4 weeks after the resumption of x-irradiation). No variations in the average level are to be noted in the males, as related to diet. In the case of the females, the results for the group on diet 4 appear to be slightly less satisfactory than those for the animals on diets 1 or 2.

The white cell count, which is the most sensitive index of irradiation injury, remained at the minimum value during the entire 10-week period, except that a partial recovery obtained between the 4th and the 7th week when x-irradiation was suspended.

DISCUSSION

Although the therapeutic effect of a fat diet cannot be demonstrated when exposure to x-irradiation is at a high level, there is no question but that this foodstuff serves to protect rats from repeated sublethal doses of x-irradiation. The protection occurs in spite of the fact that there is apparently no concomitant improvement in hemoglobin level or leukocyte count over that observed in rats receiving a fat-free regimen.

The factor in fat responsible for the protective action against x-irradiation would appear to be the essential fatty acids. This conclusion is based upon two considerations. In the first place, the essential fatty acids are the only known required components of fat not included in the basal diet. In the second place, the fact that protection occurs when cottonseed oil is given at a 2% level in the diet supports this hypothesis. On the basis of an average consumption of 10 gm

of food daily, this would account for an intake of 200 mg of cottonseed oil or 100 mg of essential fatty acids per day. In the tests in series III there was a slightly lower average mortality for the males on the 30% fat diet than for those on the 2% fat regimen. This may possibly be ascribed to the fact that the essential fatty acid intake was at a suboptimum level on diet 2. In fact, our recent results (Deuel et al., '51) indicate that, in male rats, the optimum level for linoleate is at least 200 mg per day or higher. The fact that as effective protection results from the 2% fat diet as from the 30% fat regimen in the case of females may well be a reflection of the markedly lower essential fatty acid requirement for this sex (Deuel and Greenberg, '50; Greenberg and Deuel, '50; Anisfeld et al., '51).

The sex variation in susceptibility to x-irradiation which was noted in series III but not in series I or II may be related to the difference in fat metabolism of the two sexes — a fact which has been repeatedly demonstrated. Although little variation was noted in the mortality levels for male and for female rats on either of the fat-containing diets (diets 2 or 4), the survival rate of the male rats on the fat-free diet was much lower than that of the females on the same dietary regimen. Thus, one can interpret such data as proving that fat has a sparing action on the male rat subjected to x-irradiation, but that this protective action is much less in the case of females.

The reason for the sex differences in resistance to x-irradiation is not evident. It may be related to the lower linoleate requirement mentioned earlier, or to a variation in the intermediary metabolism of fat. Such differences have been reported in the fasting ketonuria of the human subject (Deuel and Gulick, '32) and in that of rats with fatty livers (Deuel et al., '37), as well as in the exogenous ketonuria of rats and guinea pigs (Butts and Deuel, '33). Similar differences have also been noted in the case of carbohydrate, since a more rapid disappearance of glycogen obtains in the female (Deuel et al., '34).

There is no immediate explanation of the fact that fat exerted a protective effect on both males and females in series II, while its effect was noted chiefly in the case of the males in series III. If differences are to be ascribed to variations in fat metabolism, one would expect these to be minimum for the older rats used in series II, while they would be accentuated in the tests on growing rats recorded in series III.

It is believed that the present experiments demonstrate that the essential fatty acids may be related to protection from irradiation injury. Since the water-soluble vitamin intake was at a greater than optimum level and the protein content of the diets was high (25 to 34%), it is difficult to see how our results could be ascribed to variations in these dietary factors. Moreover, inasmuch as the water-soluble vitamins and proteins were present in constant proportions per 100 Cal. of diet, the rats would be expected to receive uniform quantities of these factors, irrespective of the diet consumed. Finally, the fact that beneficial effects in decreasing irradiation injury were noted in the case of rats on the 2% cottonseed oil diets would seem to indicate that a variation in consumption of water-soluble vitamins or proteins is not the explanation for the therapeutic action of fat diets.

Although the evidence seems to indicate that the protective action of fat is direct, one may still postulate that the presence of fat in the diet has facilitated a more satisfactory utilization of some other dietary component. Thus, by this indirect means, the vitamins or proteins would become responsible for the protection from x-irradiation injury. There are at present no data in support of this latter hypothesis.

SUMMARY

No differences were noted in the susceptibility of rats to x-irradiation injury as related to the fat content of the diet when the animals (on the diets for three to 4 weeks) were subjected to 650 or 850 *r* in a single dose.

On the other hand, when rats (on the diets for 12 weeks) were treated with a series of sublethal doses of x-irradiation

at weekly intervals (2,400 *r* over 10 or 14 weeks in 300 *r* doses), the rats receiving fat in their diets were found to be more resistant, and survived over a longer interval than did those on the fat-free regimen.

The protective effect of fat was approximately as satisfactory when cottonseed oil was incorporated in the diet at a 2% level as when it comprised 15 or 30% of the diet. The beneficial action of fat is therefore believed to be related to its content of essential fatty acids.

In one series of tests on young rats (two to three months old), the protective effect of fat was evident only in the male rats and not in the female rats. However, in a test with older rats (18 months), both sexes were protected to an equal extent by the incorporation of fat in the diet.

In spite of the beneficial results produced by fat in lowering the mortality rate of rats following exposure to x-irradiation, no concomitant improvements were noted in such indices of x-irradiation injury as hemoglobin level or leukocyte count.

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INTRAVENOUS INJECTION OF FAT-SOLUBLE VITAMINS AND TRANSFER TO MILK OF INJECTED VITAMIN A [1]

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THREE FIGURES

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The purpose of this investigation was to study the transfer to milk of vitamin A dispersions injected intravenously as compared with that following oral doses of oily and aqueous vitamin A.

Previous studies have indicated that mammary transfer of vitamin A is a function of serum level (Sobel et al., '50). From this it should follow that maximal transfer of vitamin A should be produced by injecting a given dose intravenously, since the highest blood levels are produced in this manner. For this reason, intravenous injections would be of value when oral feeding is not practical, and of marked advantage in favoring diffusion across impaired body membranes by providing high concentration gradients from the blood to a given tissue. Moreover, in animal husbandry, intravenous injections would be a more certain method than oral feeding in overcoming the fat-soluble vitamin deficiencies occurring in ruminants and horses (Oser, '48; Harris, '49).

Since the dispersing agent in aqueous dispersions (Sobel et al., '48) causes hemolysis (Krantz et al., '48; Sobel et al., '49), we determined the maximum concentrations of the dispersing agent tolerated without hemolysis.

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EXPERIMENT ON HEMOLYTIC ACTION OF DISPERSING AGENT

Ten grams of sorethytan laurate and 1.25 gm of oily vitamin A concentrate, dispersed in 100 ml of 0.85% NaCl solution, were added in increasing amounts to 0.5 ml of heparinized whole blood and the mixture was then incubated at 37°C. for 24 hours. Dilution to 10.0 ml was made with Ringer's solution and this was followed by separation of the intact red cells by centrifugation. One milliliter of the supernatant layer was diluted to 10.0 ml with distilled water.

TABLE 1

Per cent hemolysis of heparinized whole rabbit blood after incubation at 37°C. for 24 hours with sorethytan laurate plus vitamin A concentrate

ANIMAL	CONCENTRATION OF SORETHYTAN LAURATE					
	0 mg %	*200 mg %*	*300 mg %*	*400 mg %*	*600 mg %*	*800 mg %*
Rabbit no. 1	0	0	2	18	26	49
Rabbit no. 2	0	0	2	12	27	51
Rabbit no. 3	0	0	2	11	29	55
Rabbit no. 4	0	0	3	15	31	61
Rabbit no. 5	0	0	4	15	34	57
Rabbit no. 6	0	0	2	9	29	58
Mean	0	0	3	14	29	56

Optical density was then read at 548 mμ in a Coleman Spectrophotometer. Conversion into per cent hemolysis was made through calibration curves prepared by hemolyzing 1.0 ml of each blood specimen with 19.0 ml of distilled water and diluting 0.1, 0.2, 0.3, and 1.0 ml aliquots to 10.0 ml with distilled water (10, 20, 30, and 100% hemolysis, respectively). The results are shown in table 1. The red cells in 100 ml of heparinized whole blood resisted as much as 200 mg of sorethytan laurate, and with 300 mg, only 2 to 4% hemolysis occurred.

Once the above conditions were established, we prepared and administered aqueous dispersions in such a manner that only 30 to 40 mg of the dispersing agent were present in 100 ml of blood, thus allowing a wide safety margin.

TOXICITY OF AQUEOUS DISPERSIONS OF FAT-SOLUBLE VITAMINS

After receiving 6 injections of 400 μg of vitamin A per pound of body weight, 4 of the test animals (rabbits) were sacrificed. Gross examination and microscopic survey of vital organ sections revealed no pathology, nor was any hemolysis observed in the blood. An examination of serum for total lipid content, cholesterol esters (Zuckerman and Natelson, '48), free cholesterol (Sobel and Mayer, '45), and urea (Sobel and Hirschman, '47), and of liver for vitamin

TABLE 2

Post-injection analyses of rabbit tissues

RABBIT	BODY WEIGHT	TOTAL LIPIDS [1]	TOTAL CHOL. [1]	FREE CHOL. [1]	FREE CHOL. [1]	UREA [1]	LIVER VIT. A	HEMATO-CRIT
	lb.	*mg %*	*mg %*	*mg %*	*%*	*mg %*	*μg*	
1	8.2	223	106	29.8	28.1	19.5	1690	0.31
2	8.8	187	84.5	21.4	25.3	16.0	1740	0.29
3	10.8	200	84.5	21.4	25.3	15.8	2440	0.32
4	9.8	503	297	76.4	25.7	..	7820	0.34
Mean	9.4	278	143	37.2	26.1	17.1	3423	0.32

[1] Serum.

A (Sobel et al., '48) revealed no pathology (table 2). An additional 32 rabbits receiving injections appeared in good health. There were no deaths, whereas control animals died from random infections during this period.

VITAMIN A TRANSFER TO MILK FOLLOWING INJECTION OF VITAMIN A

It became evident that the production of high blood levels of fat-soluble vitamins by intravenous injection of aqueous dispersions is practical. We next proceeded to compare mammary transfer following intravenous injection of an aqueous vitamin A dispersion with that following oral administration of the same vitamin A product.

Five dairy cows received by jugular vein 0.1 ml per pound of body weight of a dispersion containing 230 μg of vitamin A per 0.1 ml 0.85% NaCl. Blood was collected prior to

the injection followed by specimens three, 30 and 60 minutes after the injection. All of the injected vitamin was recovered in the three-minute post-injection blood sample. Milk was collected for 24 hours prior to the dose and 48 hours thereafter, and examined for vitamin A content (Sobel and

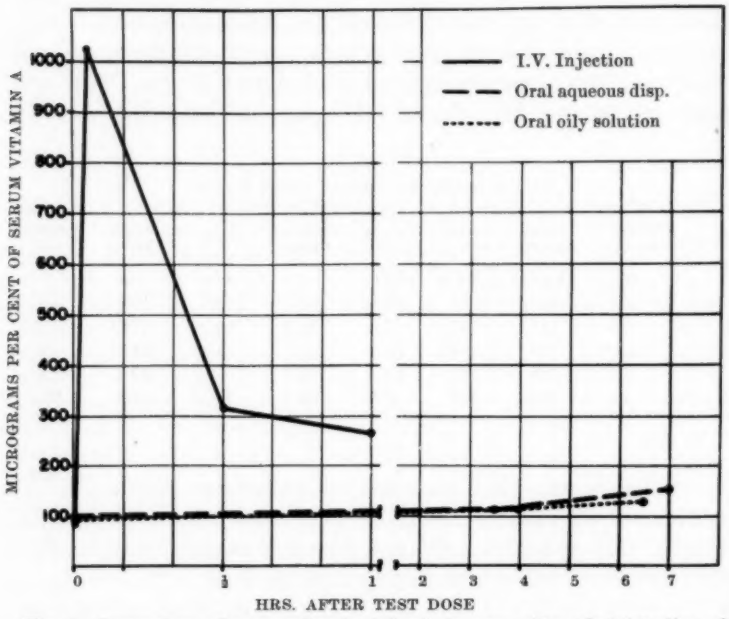

Fig. 1 Comparison of serum vitamin A levels in cows after administration of test dose by intravenous injection, aqueous dispersion orally, and oily solution orally.

Rosenberg, '49). The same dose was given orally to three additional cows by quantitative rinsing through a stomach tube. (One of the three cows given aqueous dispersions orally was eliminated from this series because there was no change in milk levels during the 50 hours after the test dose.) Blood was collected prior to the dose and also three and one-half, 4, and 7 hours thereafter, and the samples were analyzed for vitamin A (Sobel and Snow, '47). The same method of collect-

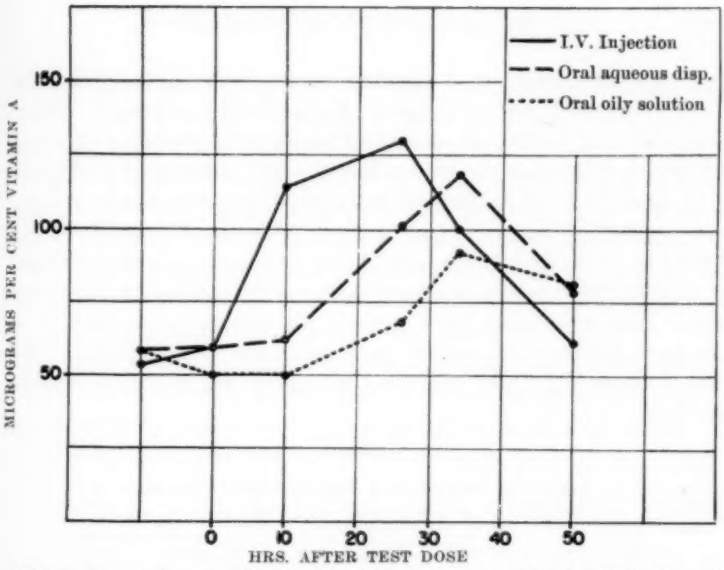

Fig. 2 Comparison of milk vitamin A levels in cows after administration of test dose by intravenous injection, aqueous dispersion orally, and oily solution orally (mean values).

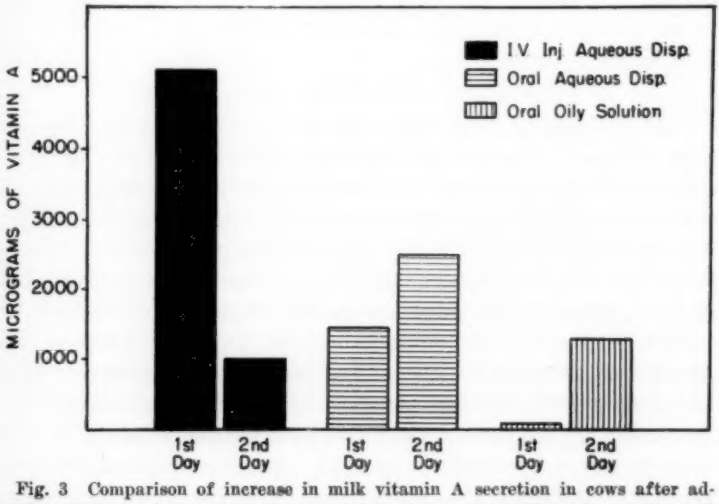

Fig. 3 Comparison of increase in milk vitamin A secretion in cows after administration of test dose by intravenous injection, aqueous dispersion orally, and oily solution orally.

ing milk was used. As a further comparison, three additional cows received the same vitamin A dissolved in corn oil. Blood was collected before the dose and again three and one-half and 6½ hours following the dose. Results are compared in figures 1, 2, and 3. They indicate that intravenous injections are far more effective in producing greater vitamin A transfer from blood to milk, and do so in a shorter time than when oral administration is made of either aqueous dispersion or oily solution. Transfer with the aqueous dispersion given orally is greater and occurs sooner than with the oily solution. The mean volume of milk secreted in liters per cow is given in table 3.

TABLE 3

Amount of milk secreted (mean) in liters per cow

HOURS	HOURS BEFORE TEST DOSE		HOURS AFTER TEST DOSE			
	10	0	10	26	34	50
Intravenous injection	3.6	4.3	3.3	4.8	3.3	4.3
Oral aqueous	3.5	2.5	2.8	3.8	2.5	3.9
Oral oily	2.5	2.0	1.9	2.6	2.4	2.3

DISCUSSION

The mechanisms that regulate the composition of milk are still obscure. One of the limiting factors that can be postulated is the transport to the site of milk production of those components of milk that are not manufactured locally. Theoretically, the diffusion of a substance like vitamin A from the blood across a membrane would be proportional to the concentration gradient of the diffusing substance.

The approximate computation is $A = KC$ where $A =$ amount of vitamin A diffusing through a unit area per unit time; $C =$ difference in concentration of vitamin A on the two sides of a given membrane, i.e., serum vitamin A and filtrate across capillaries; and $K =$ constant depending upon type and thickness of membrane. A more exact presentation is given by Davson and Danielli ('43).

Our previous studies in humans indicate that such considerations apply to vitamin A transfer from blood to milk (Sobel et al., '50). When higher blood levels were obtained milk levels followed. Since blood levels are maximal following intravenous injection (fig. 1), milk levels should be higher and reach an earlier maximum than with orally ingested aqueous dispersions. This was so when milch cows received (1) intravenous injections of vitamin A, (2) oral doses of the same dispersion, and (3) oral doses of the same vitamin A in oil. The increase in milk vitamin A for 24 hours after the dose was given was 15 times as great following the injection, and was 5 times as great with oral doses of the aqueous dispersion as with oily solutions given orally. The maximum, following the theory, took place much earlier with intravenous injection than with oral administration (figs. 2 and 3). This marked increase in transfer of vitamin A is important where deficiency may exist in one or more tissues due to impaired membranes of cell and capillary walls. Intestinal membrane offers an analogy. Vitamin A deficiency may result when this membrane is defective or injured, or the chemical system necessary for fat absorption is not functioning (May and Lowe, '48; Popper et al., '47; Kramer et al., '47). A similar situation may exist in fat-soluble vitamin transfer from blood to a tissue. This may be the reason why a variety of skin diseases displaying hyperkeratosis improve after prolonged treatment with high vitamin A doses, which provide the high blood levels probably needed to overcome resistance of impaired membrane systems, and allow a sufficient amount of the vitamin to reach the affected tissue (Harris, '49). In acne vulgaris, earlier improvement was obtained with the high blood levels following aqueous dispersions (Davidson and Sobel, '49) than reported by Straumfjord ('43) who gave twice the dosage in oil. Intravenous injections, by-passing possible intestinal absorption barriers, produce immediate maximal blood levels of controlled concentration, and should prove highly effective in

the treatment of local tissue deficiency of the fat-soluble vitamins.

A great deal of evidence in the recent literature indicates that the lack of sufficient fat-soluble vitamins, especially of vitamin A, is a problem not only in human nutrition, but also in animal husbandry in raising ruminants and horses (Harris, '49; Oser, '48). The existence of vitamin A deficiency in dairy cows on normal rations has been emphasized by Tom ('47) and again by Alvarez ('47), who noted corneal ulceration and interference with gestation due to vitamin A deficiency. Madsen and Earle ('47) reported the condemnation of 651 beef carcasses for edema due to vitamin A deficiency. Madsen et al. ('48) further reported that fertility in bulls is established only when sufficient vitamin A is received. In the absence of this supplement, rapid decline of sperm motility and sexual activity follows. Sterility in horses was overcome by the parenteral administration of vitamin A (Ribeiro and Janz, '46). Scours is a disease of newborn calves in which vitamin A deficiency is a factor. Since the young calf receives its vitamin A from the mother's milk, the remedy lies essentially in increasing the vitamin A content of the milk secreted (Oser, '48; Wise et al., '46). Administration of vitamin A by mouth fails due to the distinct impairment of vitamin A absorption that exists in calves with scours (Sellers et al., '49). The intravenous administration of the fat-soluble vitamins may be most practical for the large ruminants, since it would assure that the animal actually obtains the complete dose and conditions for transfer to milk and to those organs of the body which need these vitamins is maximal.

SUMMARY

Milch cows were given (1) intravenous injections of an aqueous vitamin A dispersion, (2) the same dispersion orally, and (3) the same vitamin A dissolved in corn oil. Increased milk vitamin A following the dose was 15 times as great with intravenous injection and 5 times as great with an oral

dose of the aqueous dispersion as with an oily solution given orally (oral doses were given through a stomach tube). The maximum transfer took place much earlier with intravenous injection than with oral administration. This is in harmony with the theory of diffusion, since maximum blood levels are reached immediately and are higher following intravenous injections than with oral doses. After repeated injection of the aqueous dispersion into rabbits, gross examination, microscopic survey of vital organ sections, and chemical analysis revealed no pathology.

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DIMINISHED URINARY CREATININE IN VITAMIN E DEFICIENT RATS[1]

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The urinary excretion of creatinine by rabbits deficient in vitamin E was shown to be unchanged, except in terminal stages, by Mackenzie and McCollum ('40), while creatine excretion rose sharply. These findings relative to the creatinine and creatine excretion by rabbits have been confirmed many times. However, some evidence has been obtained (Hove, '49) that rats deficient in vitamin E show a somewhat different excretion pattern, in that creatinine diminishes to nearly the same degree as the creatine increases. Data to confirm and extend this observation are presented in this paper.

EXPERIMENTAL

Male rats of Sprague-Dawley origin were reared from weaning (average body weight 48 gm) on diets composed of: water-washed casein (Salmon, '47) at 10% or 18%; salt mixture (Salmon, '47), 4%; vitamin pre-mix, 10%; lard, 9%; cod liver oil, 1%; and sucrose to 100%. The vitamin pre-mix contained pure vitamins mixed with sucrose to furnish the following levels per gram of diet: thiamine, riboflavin and pyridoxine,

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5 μg each; calcium pantothenate, 25 μg; niacin, 50 μg; choline, 2 mg; *i*-inositol, 0.2 mg; and 2-methyl 1–4 naphthoquinone, 0.5 μg. Approximately half of the rats in each of the several groups received the diets to which 0.01% DL-α-tocopherol acetate had been added. The animals were housed individually and fed ad libitum.

TABLE 1

Diminished creatinine excretion by vitamin E-deficient rats

CASEIN LEVEL IN THE DIET	TIME	NO. OF RATS	AVER. BODY WT.	DIETARY VITAMIN E (0.01%)	URINE CREATINE	URINE CREATININE [1]			TOTAL AS CREATININE [2]
					Aver.	Aver.	S.E.	*t*	
%	*months*		*gm*		*mg per day per kg of body weight*				
18	2	6	230	—	4.2	22.4			26.0
	2	4	235	+	2.6	24.8			27.0
	8	14	391	—	14.3	19.8	0.93	4.16	32.1
	8	12	461	+	4.2	26.3	1.26		30.0
	20	16	336	—	16.6	19.0	0.95	4.63	33.3
	20	14	437	+	3.1	24.1	0.55		26.8
10	1	5	101	—	9.4	31.8			39.9
	1	5	110	+	4.3	32.3			36.0
	2	10	145	—	22.0	23.1	1.32	2.60	42.1
	2	10	164	+	5.6	29.5	2.08		34.3
	4	15	215	—	14.9	18.7	0.76	8.40	31.5
	4	14	227	+	2.4	28.6	0.90		30.7

[1] Standard error of the mean (S.E.) was derived from the formula: $S.E. = \sqrt{S\,d^2/n(n-1)}$.

The value of *t* was derived from the formula: $t = \frac{D}{\sqrt{SE^2_1 + SE^2_2}}$.

[2] The total included the creatinine plus the creatine/1.16.

At the time intervals indicated in table 1, 24-hour urine samples were collected and diluted to 100 ml. Creatinine was determined by the alkaline picrate method as outlined by Hawk, Oser, and Summerson ('49). Creatine was determined by the direct method of Ennor and Stocken ('48).

The results given in table 1 show that the development of a vitamin E deficiency in rats, as indicated by the increased creatine excretion, was associated with a highly significant

decrease in creatinine excretion, and with a tendency for the total of these two components, expressed as creatinine, to be constant. All of the rats on the 18% casein diet deficient in vitamin E for 20 months showed signs of muscular dystrophy on gross examination. Three of the rats had severe dystrophy with the entire hind-quarters functionless, 9 had moderately severe dystrophy, while the remaining 4 had only slight dystrophy as shown by laxness in the hind legs after manual extension. None of the other animals reported in the table showed gross evidence of dystrophy.

Rats on the diet with 10% casein developed a marked creatinurea and a concomitant decline in creatinine excretion by the end of two months, while at this time the animals on the higher casein diet showed little, if any, evidence of deranged creatine metabolism. These results reaffirm the importance of the casein level on the development of vitamin E deficiency symptoms. An influence of the dietary casein level on the vitamin E requirement of rats was shown by Hove ('46) and confirmed by Hove and Harris ('47), Moore ('49), and Granados, Aaes-Jorgensen and Dam [3] ('50).

In table 2 are given the correlation coefficients between creatine and creatinine for the major groups of animals studied. For 55 animals deficient in vitamin E the correlation coefficient r was -0.36 between urinary creatinine and creatine. This correlation is significant at the 1% confidence level, and indicates that the analytical variations within this group reflect different degrees of the deficiency state of the animals. On the other hand, the coefficient of correlation between urinary creatine and creatinine of the 50 rats receiving vitamin E was -0.19. This falls short of statistical significance and indicates that the variations in the analytical values may be attributed to analytical error or random biological varia-

[3] These authors apparently were under the impression that Hove ('46) reported complete protection against all symptoms of a vitamin E deficiency when the casein level was raised to 40%. While this level of casein did prevent depigmentation (i.e., complete whitening) of the upper incisors, it is immediately evident from the data that even this level did not prevent partial de-colorization.

bility. When the groups with and without vitamin E were combined and the coefficient of correlation between creatine and creatinine of all 105 rats calculated, the resulting r was -0.59. This result seemed mathematically impossible and

TABLE 2

Correlation coefficients between creatinine and creatine excretion values of the rats included in table 1 [1]

GROUP	NO. OF RATS	CORRELATION COEFFICIENT [2] BETWEEN CREATINE AND CREATININE	VALUE OF r NECESSARY FOR SIGNIFICANCE AT THE 1% PT.[3]
All vitamin E deficient rats	55	-0.36	0.354
All rats with vitamin E	50	-0.19	0.372
All rats	105	-0.59	0.254

[1] The small groups at one month on the 10% casein diet and two months on the 18% casein diet were omitted from these calculations.

[2] Calculated from the formula $r = Sxy/Sx^2 \cdot Sy^2$ where x and y are the deviations from the means.

[3] From page 149 of *Statistical Methods*, 4th edition, 1946, by G. W. Snedecor, The Iowa State College Press, Ames.

TABLE 3

Muscle and urine creatine of rats on 10% casein and 18% casein diets (Each group had 7 male rats maintained on the diets for 8 weeks from weaning)

DIETARY LEVEL		AVER. WEIGHT GAIN IN 8 WKS.	AVER. MUSCLE CREATINE (dry basis)	AVER. URINE CREATINE DAILY
Casein	α-Tocopherol			
%	%		*mg/gm*	*mg/kg*
10	0.01	120	17.8 ± 0.66 [1]	5.4
18	0.01	171	15.3 ± 0.54	2.3
10	0	102	15.4 ± 0.80	20.9
18	0	177	14.1 ± 0.46	2.8

[1] Standard error of the mean.

therefore was adequately checked. However, it reflects merely the wide separation between the analytical values in the two groups of animals.

The seemingly higher average creatinine values of all of the rats on the 10% casein diet with vitamin E (30.1 mg/day/kg) as compared with the larger rats on the 18% casein

diet with vitamin E (25.3 mg/day/kg) (table 1) was reflected in a similar variation in muscle creatine. Rats from another experiment after 8 weeks on the diet showed the results given in table 3. Muscle creatine was significantly higher in rats on the lower casein diet; this may account for the higher creatinine excretion. The reason for the higher muscle creatine in such rats is not clear. It may simply reflect a lesser concentration of muscle cells in the larger animals due to greater quantities of intramuscular fat and fibrous tissue.

DISCUSSION

The relative immediacy of the need for vitamin E by the growing rat and rabbit probably relates to different metabolic processes in these species. It was not surprising, therefore, to note a different pattern in the excretion of creatine and creatinine.

The data indicate that in the vitamin E deficiency in rats the creatinine excretion decreased significantly. This decrease in creatinine excretion correlated negatively with the increased excretion of creatine and tended to minimize the total loss of creatine plus creatinine. It should be noted that the diet contained no added folacin, vitamin B_{12}, sulfur amino acids, or yeast. These are involved in some way with vitamin E metabolism, possibly through creatine synthesis in the liver of the animal. The growth increment in rats due to vitamin E additions to a low-protein diet can be replaced by vitamin B_{12}, especially when combined with folacin (Hove and Hardin, '51b), or by a yeast preparation or xanthine (Hove and Harris, '47). Gyorgy and Goldblatt ('49) state that sulfur amino acids replace vitamin E in protecting rats against fatal liver necrosis. Hardin and Hove ('51) reported that complete protection against methionine toxicity in young rats was prevented only when vitamin E and folacin were included in the diet along with glycine and arginine. Conversely, glycine toxicity was prevented by a combination of methionine and vitamin E. These facts support previous evidence

of the involvement of vitamin E in creatine synthesis (Hove and Hardin, '51a).

A diminished creatinine excretion in progressive muscular dystrophy of humans was emphasized by Hoagland et al. ('45). These workers noted that the decreased creatinine excretion counterbalanced the increased creatine excretion in this condition. However, the dystrophy in humans cannot be cured by simple vitamin E therapy. Scott ('51) reported studies on the enlarged hock disorder in turkeys, a condition said to resemble muscular dystrophy in other animals and strongly suspected of involving vitamin E in some way. While vitamin E was only slightly protective against this disease, the data do show that creatinine excretion in the absence of vitamin E was low. The average value, without vitamin E in the diet, was 10.8 mg/day/kg, and with vitamin E supplements it was 19.2 mg/day/kg.

The data presented herein seem consistent with the hypothesis that creatinine excretion diminishes in those species of animals in which the vitamin E deficiency assumes a chronic state but does not change in animals such as the rabbit in which the need for vitamin E is so urgent that death ensues after only a few weeks of its absence from the diet.

SUMMARY

Rats on diets deficient in vitamin E excreted less creatinine than did control rats receiving this factor. The diminished creatinine excretion correlated significantly with the increased creatine excretion of the deficient animals.

On the 10% casein diet, without vitamin E, rats developed the low creatinine-high creatine pattern much sooner than rats on the 18% casein diet.

ACKNOWLEDGMENT

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THE EFFECT OF VITAMIN B_{12} UPON THE UTILIZATION OF CHOLINE AND BETAINE BY THE YOUNG POULT

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TWO FIGURES

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The relationship between vitamin B_{12} and methylating compounds in the chick has been demonstrated by Schaefer et al. ('49) and Gillis and Norris ('49). The latter workers also found that chicks deficient in vitamin B_{12} utilized betaine more effectively than choline, while normal chicks used either supplement equally well for growth when a diet of practical feedstuffs was employed (Gillis and Norris, '51a). Jukes and Stokstad ('51) reported that with the use of a purified diet, the choline requirement for growth was reduced by supplementing the ration with vitamin B_{12}, while the choline required to prevent perosis was not decreased.

Since the poult requirement for choline is greater than that of the chick (Jukes, '40; Evans et al., '42), it was of interest to determine the effect of vitamin B_{12} upon the utilization of choline and betaine by the poult.

EXPERIMENTAL

Three experiments were conducted in which poults were fed rations containing various combinations of vitamin B_{12}, choline chloride and betaine hydrochloride. The basal ration contained 55% of soybean oil meal which had been extracted with methanol. The remainder of the ration comprised calcium gluconate, 5.0%; calcium phosphate (tribasic), 3.0%;

cottonseed oil, 2.5%; salt mixture (Kratzer et al., '49), 2.5%; vitamin mixture [1] (Kratzer et al., '49), 2.0%; calcium carbonate, 1.5%; fish oil (2,250 A, 300 D), 1.0%; alpha-tocopherol acetate, 0.002% and cornstarch to total 100%.

Crystalline vitamin B_{12} [2] in water solution was added as a supplement to some of the rations (20 μg/kg) and each poult on a vitamin B_{12}-supplemented ration was also given an injection of 10 μg of vitamin B_{12} into the breast muscle at the start of the experiment. Pure betaine hydrochloride [2] was used as a supplement, while the choline chloride used was a commercial product which was mixed with calcium carbonate. The amount of calcium in the ration was maintained constant by varying the amount of calcium carbonate to compensate for that added with the choline chloride. By calculation (Almquist, '48), the ration contained 0.47% methionine, 0.40% cystine and 26.6% crude protein. The basal ration was thus somewhat deficient in methionine, since the poult requires approximately 0.5% (Kratzer et al., '49) in a ration containing 24% protein.

Bronze poults from hens which were depleted of vitamin B_{12} (Kratzer, '52a) were used in the first 5 groups of experiment 1 and throughout experiment 2. Poults from bronze hens fed a normal breeding ration were used for groups 6 to 10 in experiment 1 and throughout experiment 3. The poults for experiments 1 and 2 were fed a vitamin B_{12}-deficient ration for 9 days and 6 days, respectively, before they were divided into equivalent groups and fed the experimental rations. In experiment 3 the day-old poults were placed on experiment immediately without being fed a depletion ration. The three experiments were continued for 13, 15 and 19 days successively. The poults were weighed at frequent intervals and the incidence of perosis, characterized by a bending and twisting of the shank, was noted.

[1] The pteroylglutamic acid was kindly supplied by Lederle Laboratories, Inc., Pearl River, New York, through the courtesy of Dr. T. H. Jukes.

[2] Kindly supplied by Merck and Co., Rahway, New Jersey, through the courtesy of Dr. D. F. Green.

The basal rations used in each experiment were assayed for choline by the method of Horowitz and Beadle ('43), employing *Neurospora crassa*. The basal rations were found to contain 6.2, 6.2 and 4.9 mg/100 gm in the successive experiments.

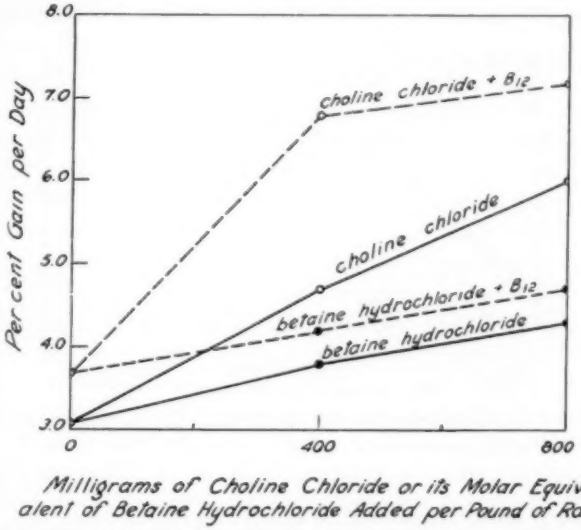

Fig. 1 Effect of choline chloride, betaine hydrochloride and vitamin B_{12} upon the growth of poults deficient in vitamin B_{12}.

RESULTS

Reasonably good agreement was noted in the gains of the poults in experiments 1 and 2, and the average percentage [3] gains of these two experiments are plotted in figure 1. The addition of choline chloride gave a straight line growth response at the levels used. When vitamin B_{12} was also added, growth increased at all levels of choline but was much greater at the 400-mg level. This indicates a sparing action of vitamin B_{12} upon the choline required for the growth of poults.

[3] Per cent gain per day $= \left(\frac{\text{Ave. gain} \times 100}{\text{Ave. wt.} \times \text{no. days}}\right)$.

Betaine hydrochloride gave only a slight growth increase at either level, and the groups receiving vitamin B_{12} gained slightly more than the controls. There was little response to vitamin B_{12} at various levels of choline when poults from normal hens were used in experiment 3 (fig. 2).

The incidence of perosis decreased as the choline in the ration was increased (table 1) but at each level the addition

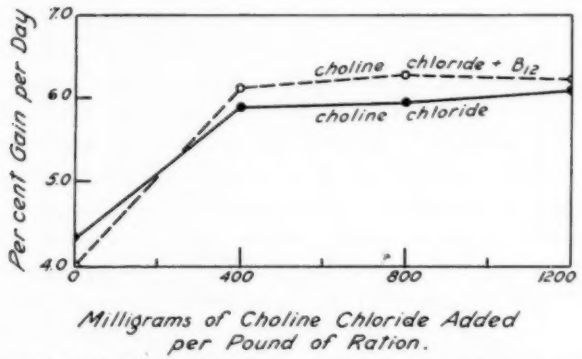

Fig. 2 Effect of choline chloride and vitamin B_{12} upon the growth of poults with normal stores of vitamin B_{12}.

TABLE 1

Effect of choline, betaine and vitamin B_{12} upon the incidence of perosis in turkey poults

SUPPLEMENT			INCIDENCE OF PEROSIS		
Choline chloride	Betaine hydrochloride	Vitamin B_{12}	Expt. 1	Expt. 2	Average (of survivors)
mg/lb.	*mg/lb.*		%	%	%
0	0	0	33	67	56
400	0	0	0	25	13
800	0	0	0	0	0
0	400	0	20	63	46
0	800	0	100	92	95
0	0	+	90	64	76
400	0	+	80	42	59
800	0	+	40	0	19
0	400	+	100	100	100
0	800	+	100	100	100
Stock mash			10		

of vitamin B_{12} caused an increase in perosis. Betaine also appeared to increase the incidence of perosis, since the highest level gave a 95% incidence. The addition of vitamin B_{12} to the diets of the groups fed betaine resulted in a 100% incidence of perosis.

DISCUSSION

The results indicate that vitamin B_{12} can spare the amount of choline required in the ration of poults to give optimum growth but that it increases the level needed for the prevention of perosis. Other functions of B_{12} are also indicated by the fact that growth responses were greater with vitamin B_{12} at all levels of choline and betaine than were observed with control birds.

The sparing action of vitamin B_{12} upon the requirement for choline for growth in poults agrees with the results obtained with chicks (Gillis and Norris, '51a; Jukes and Stokstad, '51) showing that birds which were deficient in vitamin B_{12} used choline less efficiently for growth. Gillis and Norris ('51a) discussed the possibility that vitamin B_{12} is needed for choline oxidase activity to convert choline to betaine, which can then be further metabolized. The increased oxidation of choline as a result of supplying vitamin B_{12} could presumably reduce the amount of choline available for the prevention of perosis. Gillis and Young ('51), however, were unable to find any difference in the choline oxidase activity of the livers of vitamin B_{12}-deficient and normal chicks.

Gillis and Norris ('51b) have suggested that vitamin B_{12} is necessary for the synthesis of methyl groups in chicks, as has been shown to be the case with rats. Unpublished results obtained in this laboratory indicate that vitamin B_{12} is also necessary for methyl synthesis in poults (Kratzer, '52b). The fact that both vitamin B_{12} and betaine increased the incidence of perosis might be explained by the increase in available methyl groups, although the reason for this effect is not apparent. Scott ('50) has noted an increase in perosis when betaine was added to a ration low in choline. The fact

that vitamin B_{12} caused growth greater than that of the vitamin B_{12}-deficient groups at all levels of choline and betaine indicates that there are functions of vitamin B_{12} other than its possible use in methyl synthesis. It is not possible at present to rule out the possibility that the effect of vitamin B_{12} upon growth may reside in its causing a greater use of choline for growth, thereby decreasing the amount available for prevention of perosis. This does not explain the increase in perosis caused by betaine, however.

The fact that poults from hens fed a normal ration show little response to the addition of vitamin B_{12} to their ration emphasizes the importance of using poults from hens deficient in vitamin B_{12} for studies in which vitamin B_{12} is a variable.

SUMMARY

Poults hatched from hens deficient in vitamin B_{12} were fed rations containing various amounts of choline, betaine and vitamin B_{12}. Vitamin B_{12} increased the effectiveness of choline in promoting growth but decreased its effectiveness in preventing perosis. Betaine caused only a slight growth increase on a low-choline ration. Betaine was ineffective in preventing perosis on a low-choline ration.

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FURTHER OBSERVATIONS ON THE UTILIZATION OF HOMOCYSTINE, CHOLINE AND RELATED COMPOUNDS BY CHICKS

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ONE FIGURE

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The effects of vitamin B_{12} and pteroylglutamic acid (PGA) upon the biological formation and metabolism of choline, betaine and methionine have been the subject of many recent investigations. The experimental approaches used in studying the interrelationships in this group of compounds have been diverse. Some investigators have used isotopes in work with tissue preparations or with intact rats, and others have followed the growth of rats or chicks. In a preliminary report (Jukes et al., '50) it was noted that the growth of vitamin B_{12}-deficient chicks was increased by methionine but not by homocystine with or without betaine. When vitamin B_{12} was supplied, growth responses were obtained to methionine, homocystine or homocystine plus betaine, thus indicating a role for vitamin B_{12} in the formation of methionine from homocystine by chicks. In the present investigation it was found that growth was usually produced in B_{12}-deficient chicks by adding a mixture of betaine or choline with homocystine to the B_{12}-deficient purified diet, but the response was no greater than that obtained with betaine or choline alone. Only when vitamin B_{12} was added was there an indication of an interaction between the "methylating" compounds and homocystine as shown by chick growth.

It has been shown that chicks and turkeys, in contrast to rats, are unable to use methionine, betaine or aminoethanol

alone or together as effective substitutes for choline in diets which are deficient in this substance (Jukes, '40, '41; Schaefer et al., '51). Mono- or dimethylaminoethanol can substitute for choline in promoting growth and preventing perosis in chicks on choline-deficient diets (Jukes et al., '45; Schaefer et al., '51), presumably by undergoing biological "methylation" to form choline. By including dimethylaminoethanol in a purified basal diet for chicks, it was possible to study the growth-promoting effects of various substances which could function as "methyl donors"; furthermore, the addition of dimethylaminoethanol enabled the effect of choline to be measured as a source of methyl groups independently of the chick's requirement for the dimethylaminoethanol moiety of the choline molecule. For example, the effect of choline on growth with a methionine-deficient diet containing homocystine was presumably an indication of the extent to which choline methylated homocystine to form methionine. When dimethylaminoethanol was omitted from the diets, the chicks grew comparatively slowly and showed a high incidence of perosis unless choline was added.

In a second series of experiments a diet was used containing dried peas as the main source of protein. This was lower in methionine than the purified diet which contained soybean protein. It was also deficient in vitamin B_{12} and further studies of the relationship between this vitamin and homocystine were thus made possible.

The addition of aureomycin was found in preliminary experiments to improve growth without affecting the qualitative responses to vitamin B_{12}, methionine or choline, and it was hence included in all the basal diets at a level of 20 mg/kg.

EXPERIMENTAL

Chicks were obtained from a breeding flock (Barred Plymouth Rock $\times$ New Hampshire) which was maintained on a corn-soybean basal diet, deficient in vitamin B_{12}, as described previously (Stokstad et al., '49). The chicks at hatching were segregated in groups of 10 to 12, each group with the same

average weight of 42 to 45 gm, and were kept in heated battery brooders in an air-conditioned room at 28°C. The diets were mixed at frequent intervals and the main supply was kept in a refrigerator. Several kilograms of DL-homocystine were kindly prepared by Dr. K. A. Burke using a method which was essentially that of Butz and DuVigneaud ('32) with a few modifications as follows: 500 gm of methionine were dissolved in 2 l of 18N H_2SO_4. This solution was refluxed slowly for 8 hours. After cooling to room temperature, the solution was neutralized with 15N NH_4OH until slightly acid to litmus, adding the ammonium hydroxide dropwise with rapid stirring and keeping the solution cool. The precipitate of homocystine was filtered and washed with cold water. The wet cake was then redissolved in a minimal amount of 2.5N HCl (about 1 l). This solution can be decolorized with charcoal if necessary and the homocystine reprecipitated by the addition of NH_4OH. The precipitate was again filtered and washed thoroughly with cold water. This preparation decomposed at about 265° to 268°C. and on microbiological assay showed the presence of less than 1% methionine.

Diet 1 consisted of glucose (Cerelose) 61.5 gm, soybean protein [1] 25 gm, calcium gluconate 5 gm, corn oil *plus* vitamins A, D and E (Stokstad et al., '49) 3 gm, bone ash 2 gm, salt mixture (Stokstad et al., '49) 2 gm, L-cystine 0.3 gm, dimethylaminoethanol HCl 0.2 gm, inositol 0.1 gm, aureomycin HCl 2 mg, and the following vitamin mixture: niacinamide 5 mg, calcium pantothenate 5 mg, thiamine HCl 1 mg, riboflavin 1 mg, pyridoxine HCl 1 mg, vitamin K compound (1-acetoxy-2-methyl-4-naphthyl sodium phosphate) 0.5 mg, and biotin 0.02 mg. The soybean protein was assayed for methionine content as follows [2]: 10 gm of protein were hydrolyzed by autoclaving for 16 hours in 6N hydrochloric acid and the hydrolysate was concentrated to a tar, taken up with water and treated with 1 gm of charcoal. The filtrate from this procedure was assayed for its methionine content with *S. faecalis* and the protein was thus found to contain 1.5% of methionine.

[1] Drackett Co. "Industrial Protein 220," Cincinnati, Ohio.
[2] Kindly carried out by Dr. H. P. Broquist.

This indicated that diet 1 contained about 0.38% of methionine. Diet 2 was identical with diet 1 except that dimethylaminoethanol was omitted and 0.5 mg of pteroylglutamic acid was added. Diet 3 consisted of ground dried split green peas 70 gm, glucose 14 gm, gelatin 8 gm, bone ash 3 gm, corn oil *plus* vitamins A, D and E 1 gm, sodium chloride 0.5 gm, DL-tryptophan 0.2 gm, manganese sulfate 25 mg, aureomycin HCl 2 mg, and the same vitamin mixture as for diet 1 plus pteroylglutamic acid, 0.2 mg. The peas were found to contain 0.19% of methionine by microbiological assay and 22% protein (Kjeldahl). This indicated that about 0.20% methionine was present in diet 3, assuming that gelatin contains 0.8% methionine (Block and Bolling, '51). Two groups of chicks were simultaneously fed the same diet in each experiment and the weight data represent the average of two groups in the case of each value shown. The results of the first experiment are shown in table 1. Experiment 2 was a repetition of experiment 1 except that three of the diets containing betaine were omitted because betaine seemed no more effective than choline in producing growth in the presence of homocystine, and the results are shown in table 2.

The diets were planned so that the supplements were added singly and in various combinations, thus enabling their growth-promoting effect to be measured under a variety of conditions; for example, by subtracting the weight of 151 gm obtained in chicks with the diet supplemented with homocystine, choline and PGA from that of 324 gm obtained with homocystine, choline, PGA and vitamin B_{12} (table 1) a figure of 173 gm is obtained as a measure of the response to vitamin B_{12}. In this manner the growth responses to homocystine, methionine, betaine, choline, vitamin B_{12} and PGA were measured under a number of dietary conditions and the effect on growth of combinations of these various supplements was also observed.

The diet containing glucose, soybean protein and dimethylaminoethanol may be regarded as being deficient in methionine, "labile methyl," vitamin B_{12} and folic acid. The deficiency

TABLE 1

Results of experiment 1 in which diet 1 was fed to duplicate groups of 12 chicks each together with various supplements

ADDITION PER KILOGRAM OF BASAL DIET	25-DAY WEIGHT	FEED CONSUMED PER DAY	INCREASE IN AVERAGE WEIGHT (IN GM) FOLLOWING ADDITION OF							
			Homocystine	Betaine + homocystine	Choline + homocystine	Methionine	Betaine	Choline	B_{12}	PGA
	gm	*gm*								
None	75 ± 4.2	9.2								
DL-homocystine (6 gm)	74 ± 5.6	10.3	— 1							
Homocystine + betaine (2 gm)	119 ± 7.7	11.3	11	44 [1]			45 [1]			
Homocystine + choline (2 gm)	140 ± 7.3	14.4	— 1		65 [1]			66 [1]		
DL-methionine (6 gm)	176 ± 8.3	15.3				101 [1]				
Methionine + choline	200 ± 6.9	17.5				59 [1]		24 [1]		
Betaine	108 ± 5.9	5.9					33 [1]			
Choline	141 ± 6.9	13.9						66 [1]		
PGA (5 mg)	92 ± 7.1	9.3								17
Homocystine + PGA	88 ± 6.1	9.9	— 4							14
Homocystine + betaine + PGA	144 ± 9.2	14.4		52 [1]			56 [1]			25 [1]
Homocystine + choline + PGA	151 ± 12.0	14.2	— 4		59 [1]			63 [1]		11
Methionine + PGA	202 ± 12.1	18.7				110 [1]				26
Choline + PGA	155 ± 10.8	14.7						63 [1]		14
B_{12} (50 μg)	182 ± 10.5	16.0							107 [1]	
Homocystine + B_{12}	242 ± 15.0	20.7	60 [1]						168 [1]	
Homocystine + betaine + B_{12}	296 ± 13.1	21.3		114 [1]			54 [1]		177 [1]	
Homocystine + choline + B_{12}	315 ± 11.0	22.5	82 [1]		133 [1]			73 [1]	175 [1]	
Methionine + B_{12}	312 ± 8.6	22.5				130 [1]			136 [1]	
Choline + B_{12}	233 ± 17.0	21.2						51 [1]	92 [1]	
B_{12} + PGA	255 ± 12.7	20.1							163 [1]	73 [1]
Homocystine + B_{12} + PGA	321 ± 12.6	22.5	66 [1]						233 [1]	79 [1]
Homocystine + betaine + B_{12} + PGA	338 ± 12.1	22.5		83 [1]			17		194 [1]	42 [1]
Homocystine + choline + B_{12} + PGA	324 ± 12.8	22.5	69 [1]		69 [1]			3	173 [1]	9
Methionine + B_{12} + PGA	349 ± 12.9	22.6				94 [1]			147 [1]	37 [1]
Choline + B_{12} + PGA	255 ± 7.8	20.0						0	100 [1]	22

[1] Significant at the 5% level.

TABLE 2

Results of experiment 2 in which diet 1 was used. Most of the supplements were identical with those in experiment 1

ADDITION PER KILOGRAM OF BASAL DIET	25-DAY WEIGHT	FEED CONSUMED PER DAY	INCREASE IN AVERAGE WEIGHT (IN GM) FOLLOWING ADDITION OF							
			Homocystine	Betaine + homocystine	Choline + homocystine	Methionine	Betaine	Choline	B_{12}	PGA
	gm	*gm*								
None	65 ± 2.8	9.0								
DL-homocystine (6 gm)	68 ± 5.5	9.5	3							
Homocystine + betaine (2 gm)	124 ± 6.0	10.5	4	59[1]			56[1]			
Homocystine + choline (2 gm)	105 ± 4.6	9.9	−8		40[1]			37[1]		
DL-methionine (6 gm)	183 ± 9.7	16.3				118[1]				
Methionine + choline (2 gm)	200 ± 10.9	17.1				87[1]				
Betaine (2 gm)	120 ± 6.7	9.8					55[1]			
Betaine (4 gm)	121 ± 4.8	9.8					56[1]			
Choline (2 gm)	113 ± 6.4	9.6						48[1]		
Choline (4 gm)	145 ± 8.9	11.1						80[1]		
PGA (5 mg)	73 ± 4.8	9.3								8
Homocystine + PGA	69 ± 6.2	9.9	−4							1
Homocystine + choline (2 gm) + PGA	133 ± 7.9	15.6	−17		60[1]			64[1]		28[1]
Methionine + PGA	162 ± 12.5	16.0				89[1]				−21
Choline (2 gm) + PGA	150 ± 7.9	15.2						77[1]		37[1]
B_{12} (50 μg)	185 ± 9.5	15.2							120[1]	
Homocystine + B_{12}	222 ± 10.4	17.1	37[1]						154[1]	
Homocystine + choline (2 gm) + B_{12}	305 ± 10.2	19.5	74[1]		120[1]			83[1]	200[1]	
Methionine + B_{12}	279 ± 9.7	18.8				94[1]			96[1]	
Choline (2 gm) + B_{12}	231 ± 8.7	16.3						46[1]	118[1]	
B_{12} + PGA	247 ± 9.0	19.0							174[1]	62[1]
Homocystine + B_{12} + PGA	282 ± 11.6	19.0	35[1]						213[1]	60[1]
Homocystine + choline (2 gm) + B_{12} + PGA	311 ± 11.0	19.0	78[1]		64[1]			29	178[1]	6
Methionine + B_{12} + PGA	317 ± 9.6	20.0				70[1]			155[1]	38[1]
Choline (2 gm) + B_{12} + PGA	233 ± 11.2	16.6						−14	83[1]	2

[1] Significant at 5% level.

of methionine was not acute because the diet contained about 0.4% of methionine and 0.3% cystine was added. These figures may be compared with the level of 0.5% methionine in the presence of 0.4% cystine estimated by Almquist ('47) to be required for the growth of chicks.

On this basal diet the growth data obtained with various supplements indicated the following:

1. No growth response was obtained to homocystine when added singly to the basal diet in the absence of vitamin B_{12}.
2. In the presence of vitamin B_{12}, homocystine produced a growth response with or without the addition of folic acid.
3. No response was obtained to choline or betaine when vitamin B_{12} and folic acid were both added. Perhaps this indicates that the presence of these two vitamins made other sources available, such as glycine and serine, of a "single-carbon fragment" for the methylation of dimethylaminoethanol so that dietary choline or betaine were not required.
4. In the absence of vitamin B_{12} the response to a mixture of homocystine and choline or to a mixture of homocystine and betaine was equal only to the arithmetic sum of the individual responses to homocystine, choline or betaine *when fed separately*. However, with vitamin B_{12} added the response to a mixture of homocystine and choline or to a mixture of homocystine and betaine was greater than the arithmetic sum of the responses to the components of the mixtures when fed individually. This may indicate that methylation of homocystine by choline or betaine proceeded only when vitamin B_{12} was added to the diet.
5. The response to methionine was relatively unaffected by the presence or absence of vitamin B_{12} or folic acid.
6. The response to vitamin B_{12} was greater in the presence of folic acid than in its absence; and also was greater in the presence of homocystine than in its absence when methionine was omitted.
7. The response to folic acid was variable; it was greatest in the presence of vitamin B_{12} with or without homocystine and it was diminished by adding choline. Perhaps this indi-

cates a role for folic acid in making glycine and serine available for the methylation of dimethylaminoethanol, for with preformed choline added to the diet the methylation of dimethylaminoethanol was no longer required to ensure the supply of choline for growth.

With dimethylaminoethanol omitted from the basal diet and with folic acid added, the deficiencies now become those of choline, "labile methyl," methionine, and vitamin B_{12}. This diet (diet 2) was used in experiments 3 and 4 and the results are shown in tables 3 and 4. In contrast to the results obtained on the diet with dimethylaminoethanol, responses to betaine in experiments 3 and 4 were markedly lower than the responses to choline. This failure to respond to betaine may be attributed to the dietary absence of the group $CH_3NHCH_2CH_2OH$ which the chick needs in the diet to prevent choline deficiency and which must be supplied in the form of either methylaminoethanol, dimethylaminoethanol, choline (Jukes et al., '45) ethyldimethylaminoethanol or diethylmethylaminoethanol, or be replaced by arsenocholine (Jukes and Welch, '42).

The growth responses to various supplements encountered in chicks on the purified diet with folic acid added and dimethylaminoethanol omitted were as follows:

1. Homocystine produced no response in the absence of vitamin B_{12}. It has been suggested elsewhere that dimethylaminoethanol might compete with homocystine for methyl groups (Gillis and Norris, '51). No evidence was seen for this in the present investigation, or homocystine would presumably have produced better growth responses in experiments 3 and 4 than in the corresponding groups in experiments 1 and 2.

2. The response to homocystine in the presence of vitamin B_{12} was rather small and was consistently improved by choline or betaine.

3. Responses to choline were obtained under all conditions of supplementation but the responses were greatest in the presence of vitamin B_{12}. There was markedly less response

TABLE 3

Results of experiment 3 using diet 2 which resembles diet 1 except that dimethylaminoethanol was omitted and folic acid was added

ADDITION PER KILOGRAM OF BASAL DIET	25-DAY WEIGHT	FEED CONSUMED PER DAY	INCREASE IN AVERAGE WEIGHT (IN GM) FOLLOWING ADDITION OF						
			Homocystine	Betaine + homocystine	Choline + homocystine	Methionine	Betaine	Choline	B_{12}
	gm	*gm*							
None	77 ± 2.7	8.1							
DL-homocystine (6 gm)	74 ± 3.8	8.0	— 3						
Homocystine + betaine (2 gm)	90 ± 5.2	8.5	— 17 [1]	13 [1]			16 [1]		
Homocystine + choline (2 gm)	130 ± 7.6	8.6	— 5		53 [1]			56 [1]	
DL-methionine (6 gm)	153 ± 13.4	9.9				76 [1]			
Methionine + choline	178 ± 8.9	10.2				43 [1]		25	
Methionine + betaine	166 ± 9.2	10.7				59 [1]	13		
Betaine (2 gm)	107 ± 4.0	6.9					30 [1]		
Betaine (4 gm)	129 ± 8.4	9.0					52 [1]		
Choline (2 gm)	135 ± 7.1	8.6						58 [1]	
Choline (4 gm)	149 ± 6.3	8.3						72 [1]	
B_{12} (50 μg)	165 ± 7.2	10.0							88 [1]
Homocystine + B_{12}	193 ± 6.7	10.8	28 [1]						119 [1]
Homocystine + choline (2 gm) + B_{12}	311 ± 9.4	19.7	35 [1]		146 [1]			118 [1]	181 [1]
Homocystine + betaine (2 gm) + B_{12}	191 ± 10.8	11.2	0	26 [1]			— 2		101 [1]
Betaine (2 gm) + B_{12}	191 ± 7.4	12.8					26 [1]		84 [1]
Betaine (4 gm) + B_{12}	163 ± 5.5	10.2					— 2		
Methionine + B_{12}	210 ± 8.5	15.7				45 [1]			57 [1]
Methionine + choline (2 gm) + B_{12}	251 ± 13.6	18.2				— 25		41 [1]	73 [1]
Methionine + betaine (2 gm) + B_{12}	236 ± 7.1	16.6				45 [1]	26 [1]		70 [1]
Choline (2 gm) + B_{12}	276 ± 9.2	16.9						111 [1]	141 [1]
Choline (4 gm) + B_{12}	263 ± 6.8	16.6						98 [1]	114 [1]

[1] Significant at 5% level.

TABLE 4

Results of experiment 4, in which experiment 3 was repeated

ADDITION PER KILOGRAM OF BASAL DIET	25-DAY WEIGHT	FEED CONSUMED PER DAY	INCREASE IN AVERAGE WEIGHT (IN GM) FOLLOWING ADDITION OF						
			Homocystine	Betaine + homocystine	Choline + homocystine	Methionine	Betaine	Choline	B_{12}
	gm	*gm*							
None	82 ± 4.2	8.6							
DL-homocystine (6 gm)	80 ± 5.9	8.0	— 2						
Homocystine + betaine (2 gm)	107 ± 5.1	9.4	— 4	25 [1]			27 [1]		
Homocystine + choline (2 gm)	120 ± 5.9	10.7	— 42 [1]		38 [1]			40 [1]	
DL-methionine (6 gm)	154 ± 9.6	14.3				72 [1]			
Methionine + choline	184 ± 11.4	17.6				22		30 [1]	
Methionine + betaine	159 ± 9.3	15.0				48 [1]	5		
Betaine (2 gm)	111 ± 4.9	9.3					29 [1]		
Betaine (4 gm)	120 ± 5.2	10.4					38 [1]		
Choline (2 gm)	162 ± 8.1	13.6					8	80 [1]	
Choline (4 gm)	142 ± 6.2	12.0						60 [1]	
B_{12} (50 μg)	154 ± 5.8	14.7							72 [1]
Homocystine + B_{12}	174 ± 7.0	15.0	20 [1]						94 [1]
Homocystine + choline (2 gm) + B_{12}	306 ± 11.5	20.9	30 [1]		152 [1]			118 [1]	186 [1]
Homocystine + betaine (2 gm) + B_{12}	204 ± 7.4	15.8	42 [1]	50 [1]			30 [1]		97 [1]
Betaine (2 gm) + B_{12}	162 ± 6.8	15.1							51 [1]
Methionine + B_{12}	227 ± 5.0	18.5				73 [1]			73 [1]
Methionine + choline (2 gm) + B_{12}	302 ± 8.8	20.0				26 [1]		75 [1]	118 [1]
Methionine + betaine (2 gm) + B_{12}	224 ± 10.2	20.0				62 [1]	— 3		65 [1]
Choline (2 gm) + B_{12}	276 ± 9.3	19.1						122 [1]	114 [1]

[1] Significant at 5% level.

to betaine than to choline and the greatest response to betaine was obtained on the unsupplemented basal diet.

4. The response to a mixture of homocystine and choline, or homocystine and betaine, was only equal to or was less than the arithmetic sum of the responses to homocystine, choline and betaine when fed separately, regardless of the presence or absence of vitamin B_{12}. This observation is difficult to interpret. Perhaps the growth response obtained to homocystine plus betaine in the presence of dimethylaminoethanol in experiments 1 and 2 was due to the formation of methionine and its subsequent utilization in the methylation of dimethylaminoethanol.

5. The responses to methionine were less than were encountered in experiments 1 and 2 and no response was obtained to methionine when B_{12} and choline were both added, thus leading to the conclusion that the growth-promoting effect of methionine when both B_{12} and choline were not present was partly due to the activity of methionine as a source of labile methyl groups which could also be supplied by choline.

6. The response to vitamin B_{12} was greater in the presence of homocystine than in its absence and was greatest in the presence of choline. Evidently, choline and vitamin B_{12} could not function interchangeably in promoting growth.

The incidence of perosis in the various groups in experiments 1 and 2 was low. However, in experiments 3 and 4 a high incidence of perosis was seen in certain groups and all the chicks were examined at 25 days of age and "scored" for perosis. During the examination, the individual who "scored" the chicks did not know which diets they received. The system of "scoring" which was employed consisted of assigning a value of 0, 10 or 20 to each hock joint of each bird depending on the absence of perosis (0 points), the presence of a mild but definite degree of perosis (10 points), or the presence of severe and deforming perosis (20 points). The maximum average score thus was 40 points and the results are summarized in table 5, in which it will be noted that excellent agreement was obtained between replicate treatments. Perosis

TABLE 5

Effect of various supplements on perosis in chicks on diet 2

(Columns I and II refer to experiments 3 and 4, respectively)

ADDITIONS TO DIET	AVERAGE PEROSIS SCORE AT 25 DAYS WITH														
	None			Choline						Betaine					
				2 gm			4 gm			2 gm			4 gm		
	I	II	Av.	I	II	Av.	I	II	Av.	I	II	Av.	I	II	Av.
None	3	0	2	1	1	1	3	1	2	17	20	18	8	6	7
Homocystine	7	5	6	1	0	0				15	17	16			
Methionine	7	4	6	5	4	4				7	5	6			
Vitamin B_{12}	28	37	32	4	1	2	5		5	29	35	32	32		32
Vitamin B_{12} + homocystine	34	37	36	3	4	4				32	32	32			
Vitamin B_{12} + methionine	25	29	27	2	0	1				21	29	25			

Maximum possible score = 40

was correlated with the combined presence of vitamin B_{12} and absence of choline. Betaine appeared to aggravate perosis slightly in the absence of vitamin B_{12}.

In the next series of experiments, diet 3 was used, which is closely similar to a diet used in a previous investigation for the simultaneous production of deficiencies of methionine and vitamin B_{12} (Jukes and Stokstad, '51a). Excellent agreement was obtained in the results of the two experiments on diet 3, as may be seen from tables 6 and 7. It is evident that diet 3 was markedly deficient in methionine and it was also deficient in vitamin B_{12} and partially deficient in "labile methyl." The response encountered on diet 3 were as follows:

1. Little or no response was obtained to homocystine in the absence of added B_{12} unless betaine or choline was added, in which case a good response was obtained to homocystine.

2. There was a marked response to homocystine in the presence of vitamin B_{12} which was improved only slightly by choline or betaine. Apparently the labile methyl donors present in the basal diet (perhaps in the peas) were used effectively only when vitamin B_{12} was added.

3. Regardless of the presence or absence of vitamin B_{12}, the repsonse to choline or betaine was quite small unless homocystine was added, leading to the conclusion that choline and betaine were functioning chiefly for the methylation of homocystine.

4. The responses to mixtures of homocystine and choline, or homocystine and betaine, were markedly greater than the arithmetic sum of the responses to these substances when fed alone in the absence of vitamin B_{12}. This augmenting effect of choline or betaine on the response to homocystine was somewhat less in the presence of vitamin B_{12} than in its absence.

5. The response to methionine was remarkably constant regardless of the presence of betaine or choline added separately or in addition to vitamin B_{12}.

TABLE 6

Growth of chicks in experiment 5 on diet 3 (deficient in methionine and containing added PGA) with various supplements

ADDITION PER KILOGRAM OF BASAL DIET	25-DAY WEIGHT	FEED CONSUMED PER DAY	INCREASE IN AVERAGE WEIGHT (IN GM) FOLLOWING ADDITION OF						
			Homocystine	Betaine + homocystine	Choline + homocystine	Methionine	Betaine	Choline	B_{12}
	gm	*gm*							
None	69 ± 2.0	9.2							
DL-homocystine (6 gm)	82 ± 3.1	10.1	13 [1]						
Homocystine + betaine (2 gm)	142 ± 13.0	14.7	60 [1]	73 [1]			60 [1]		
Homocystine + choline (2 gm)	165 ± 7.8	14.9	87 [1]		96 [1]			83 [1]	
DL-methionine (6 gm)	238 ± 13.1	18.8				169 [1]			
Methionine + betaine	262 ± 11.1	19.4				180 [1]	24		
Methionine + choline	247 ± 11.3	18.8				169 [1]		9	
Betaine	82 ± 2.4	10.0					13 [1]		
Choline	78 ± 2.7	9.4						9 [1]	
B_{12} (50 μg)	103 ± 3.2	10.9							34 [1]
Homocystine + B_{12}	270 ± 16.7	20.5	167 [1]						188 [1]
Homocystine + betaine + B_{12}	330 ± 14.1	20.8	217 [1]	227 [1]			60 [1]		188 [1]
Homocystine + choline + B_{12}	293 ± 16.4	20.3	181 [1]		190 [1]			23	128 [1]
Methionine + B_{12}	304 ± 13.0	20.0				201 [1]			66 [1]
Betaine + B_{12}	113 ± 4.0	12.1					10 [1]		31 [1]
Choline + B_{12}	112 ± 5.2	13.4						9	34 [1]
Methionine + betaine + B_{12}	291 ± 10.3	21.6				178 [1]	−13		29
Methionine + choline + B_{12}	327 ± 13.5	21.6				215 [1]		23	80 [1]
Methionine (2 gm) + B_{12}	274 ± 8.2	22.5				171 [1]			
Methionine (4 gm) + B_{12}	311 ± 10.5	22.5				208 [1]			

[1] Significant at 5% level.

TABLE 7

Growth of chicks in experiment 6, a repetition of experiment 5

ADDITION PER KILOGRAM OF BASAL DIET	25-DAY WEIGHT	FEED CONSUMED PER DAY	INCREASE IN AVERAGE WEIGHT (IN GM) FOLLOWING ADDITION OF						
			Homocystine	Betaine + homocystine	Choline + homocystine	Methionine	Betaine	Choline	B_{12}
	gm	*gm*							
None	74 ± 2.3	9.1							
DL-homocystine (6 gm)	85 ± 4.9	9.1	11						
Homocystine + betaine (2 gm)	151 ± 7.9	13.5	67 [1]	77 [1]			66 [1]		
Homocystine + choline (2 gm)	163 ± 6.8	14.4	77 [1]		89 [1]			78 [1]	
DL-methionine (6 gm)	217 ± 7.5	17.8				143 [1]			
Methionine + betaine	242 ± 10.4	18.1				158 [1]	25 [1]		
Methionine + choline	236 ± 13.8	18.3				150 [1]		19	
Betaine	84 ± 3.3	9.2					10 [1]		
Choline	86 ± 1.9	9.6						12 [1]	
B_{12} (50 μg)	108 ± 5.7	14.5							34 [1]
Homocystine + B_{12}	254 ± 11.0	17.3	146 [1]						169 [1]
Homocystine + betaine + B_{12}	315 ± 14.8	21.3	200 [1]	207 [1]			61 [1]		164 [1]
Homocystine + choline + B_{12}	330 ± 12.2	20.8	218 [1]		222 [1]			76 [1]	167 [1]
Methionine + B_{12}	284 ± 12.3	20.8				176 [1]			67 [1]
Betaine + B_{12}	115 ± 5.0	13.3					7		31 [1]
Choline + B_{12}	112 ± 4.7	13.9						4	26 [1]
Methionine + betaine + B_{12}	292 ± 11.7	21.0				177 [1]	−7		50 [1]
Methionine + choline + B_{12}	302 ± 15.9	20.8				190 [1]		18	66 [1]

[1] Significant at 5% level.

6. The response to vitamin B_{12} was very marked in the presence of homocystine with or without choline or betaine; it was moderate in the presence of methionine, and small on the basal diet with or without betaine or choline.

The effect of methionine and homocystine in the presence and absence of vitamin B_{12} on the growth of chicks is shown in figure 1, which strikingly illustrates the relation of vitamin

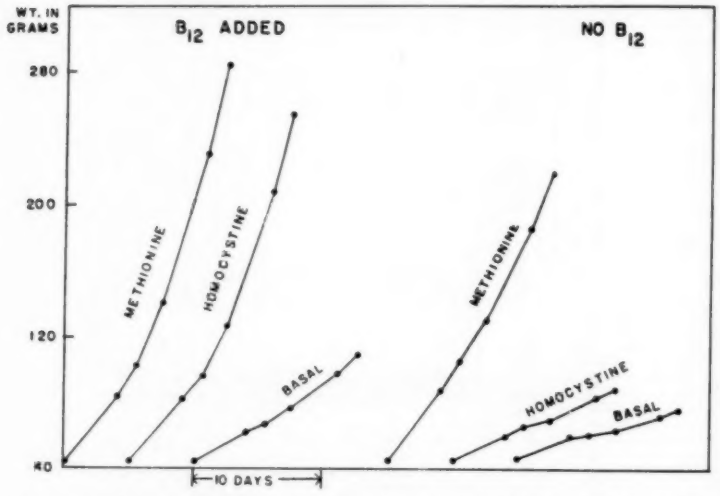

Fig. 1 Growth responses of chicks on diet 3 to methionine and homocystine, with and without vitamin B_{12}. Basal diet 3 was used (see table 7).

B_{12} to the utilization of homocystine in chicks. Evidently a factor was present in the basal diet which enabled homocystine to replace methionine for growth in the presence of vitamin B_{12}. It was thought possible that the factor in the basal diet might be choline, since Engel ('43) has reported that dried peas contain 0.29% of this substance. Evidently the choline *furnished by the basal diet* was utilized far less efficiently for the methylation of homocystine in the absence than in the presence of supplementary vitamin B_{12} as shown by the

following data which are average weights taken from tables 6 and 7.

ADDITION TO BASAL DIET	AVERAGE WEIGHT AT 25 DAYS		INCREASE DUE TO HOMOCYSTINE
	Without homocystine	With homocystine	
	gm	*gm*	
None	72	84	12
Vitamin B_{12}	106	262	156

In contrast, *added* choline seemed to be utilized to a marked extent for methylation even in the absence of vitamin B_{12} as judged by the finding that chicks on the diet supplemented with homocystine *plus* choline weighed twice as much (164 gm) as the chicks on the diet supplemented with choline alone (82 gm). When calculated similarly, the increase due to methionine was 156 gm without vitamin B_{12} and 188 gm with vitamin B_{12} in the diet, showing that the utilization of methionine was relatively unaffected by B_{12}.

Another possible explanation for the growth-promoting effect of homocystine when added to methionine-deficient diets arises from observations which have been made with rats indicating that in the presence of ample vitamin B_{12} and PGA, methylation of homocystine may take place without the addition of a dietary supply of "methyl donors" such as choline or betaine (Bennett, '50). Glycine and serine, by giving rise to formate (Sakami, '49; Siekevitz and Greenberg, '50; Shemin, '46) which could serve in turn as a precursor of the methyl group of methionine in experiments with the tissues of rats and guinea pigs (Siekevitz and Greenberg, '50; Shemin, '46; Welch and Sakami, '50) are obvious possibilities as sources of the "single-carbon" fragment which can form a methyl group. An experiment was carried out to investigate this point by extracting the ground peas with hot alcohol to remove choline and possibly betaine. Such a procedure should not affect the glycine and serine in the diet which are derived both from pea protein and gelatin. The results of the experiment are shown in table 8. It may be seen that alcohol-extraction largely removed the component of the basal diet which

was presumably responsible for the methylation of homocystine in the presence of vitamin B_{12} and PGA. Only a minor role can therefore be assigned to glycine and serine in the transformation of homocystine to methionine under the conditions of these experiments and the effectiveness of choline as a "methyl donor" is emphasized.

TABLE 8

Effect of various supplements on growth of chicks on diet 3 which was modified by extracting the ground peas with hot ethanol

ADDITION PER KILOGRAM OF BASAL DIET	AVE. WEIGHT OF CHICKS AT 25 DAYS (GM)	
	Without supplemental choline	2 gm choline added per kilogram of diet
None	68	82
DL-homocystine, 6 gm	69	144
Vitamin B_{12}, 50 μg	90	112
Homocystine + B_{12}	108	295

DISCUSSION

The ability of the chick to form methionine from choline and homocystine was first indicated by the experiments of Klose and Almquist ('41). Their chicks were placed on a "standard chick mash" for a week preceding the experiments, and this procedure makes it probable that vitamin B_{12} deficiency was not encountered.

It was found in our laboratory (Jukes et al., '50) that vitamin B_{12}-deficient chicks on a purified diet containing soybean protein would not show a growth response to either homocystine or homocystine *plus* betaine. In the present investigation vitamin B_{12}-deficient chicks on a similar diet did not respond to homocystine but responded to homocystine *plus* betaine (tables 1 to 4). The reason for this apparent disparity could well be that the chicks in the earlier investigation may have been more completely depleted of vitamin B_{12}, which is evidently needed for the methylation of homocystine by the chick. Variations in the extent of B_{12} deficiency will therefore influence the response of chicks to homocystine

and betaine, thus accounting for differences in various investigations (Jukes et al., '50; Gillis and Norris, '51).

Folic-acid deficiency in chicks has been shown to depress the formation of methionine from homocystine by liver homogenates *plus* choline or betaine (Dinning et al., '51). Similarly in rats, liver homogenates from vitamin B_{12}-deficient animals showed a lower ability to form methionine from homocystine *plus* choline or betaine as compared with homogenates from rats receiving vitamin B_{12}. The close interrelationship of folic acid and vitamin B_{12} in this and other biochemical processes affords an interesting sidelight to the circumstances surrounding the numerous observations, which are reviewed elsewhere (Jukes and Stokstad, '51b), that either folic acid or vitamin B_{12} will produce a hemopoietic response in pernicious anemia.

In the present investigation the response to pteroylglutamic acid was lowered when B_{12} and choline were both present in the diet (tables 1 and 2), perhaps indicating that these substances in combination "spare" the folic acid requirement of chicks.

SUMMARY

1. A study was made of the responses of vitamin B_{12}-deficient chicks to homocystine, betaine, choline, methionine, vitamin B_{12} and folic acid as measured by early growth on diets which were deficient in several respects. A "factorial" design of adding the supplements enabled their interrelationships to be measured.

2. The response to homocystine was markedly increased by vitamin B_{12} under a variety of dietary conditions. However, in the absence of vitamin B_{12} homocystine often actually depressed growth.

3. The response to homocystine in the presence of added vitamin B_{12} was increased by the addition of choline or betaine. However, when vitamin B_{12} was not added, the response to homocystine plus choline or betaine added as a mixture was no greater than the sum of the responses to homocystine and choline or betaine when tested individually.

4. Choline appeared to be highly effective as a "methylating" agent for homocystine in the presence of vitamin B_{12} on a diet which was markedly deficient in methionine. There was no indication that the amino acids in the basal diet could effectively replace choline for this purpose.

5. The incidence of perosis on a purified diet without added dimethylaminoethanol was high when vitamin B_{12} was added and choline was omitted. The incidence was not reduced by adding betaine.

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NUTRITIONAL EVALUATION OF FOOD PROTEINS BY MEASURING AVAILABILITY OF AMINO ACIDS TO MICROORGANISMS

I. COTTONSEED PROTEINS [1]

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To evaluate the changes which take place in a protein when a food is processed, the essential amino acid content and the change in digestibility of the protein before and after processing should be known. Many workers have shown that heat changes the nutritive value of a protein (McCollum and Davis, '15; Block et al., '34; Eldred and Rodney, '46; Patton and Hill, '48; Riesen et al., '47; Evans and Butts, '48; Pader et al., '48; Stevens and McGinnis, '47; Mader et al., '49; Olcott and Fontaine, '41; Ingram et al., '50; Dechary and Altschul, '49; Simon and Melnick, '50; Evans and Butts, '49; Hou et al., '49; Baldwin et al., '51; Hadden et al., '50). Two types of heat inactivation apparently take place; one the destruction of amino acids, the other a binding of the amino acids so that they are not liberated by digestion *in vivo* or by enzymatic hydrolysis *in vitro*, but are liberated by acid hydrolysis (Evans and Butts, '48). It is the purpose of this investigation to study these two types of inactivation on a series of foods, processed under known conditions, and to attempt to evaluate the nutritional quality of the various food proteins from the data obtained.

[1] This is the first of a series of papers on the availability of amino acids in raw and processed foods.

Considerable time was expended to develop an enzyme system by which the extent of hydrolysis could be made reproducible. It was found that by rigid control of conditions good reproducibility could be obtained.

This paper deals with results obtained on a series of cottonseed meals prepared under known conditions. Rat-feeding tests to evaluate the protein quality were carried out at the same time as microbiological assays of acid and enzyme digests, and a comparison was made of the two methods of studying protein damage. The results obtained on these meals by microbiological assay of enzyme digests agree quite well with results obtained from animal-feeding experiments using the same meals.

EXPERIMENTAL

As part of the research program inaugurated by the Southern Regional Research Laboratory, in cooperation with the cottonseed industry, a series of 10 cottonseed meals (series 1) was received which were prepared under different processing conditions, particularly the temperature and time of cooking (Hadden et al., '50; table 1). The meal used as a standard on the first series was an essentially gland-free meal (Vix et al., '49), and was prepared at the Southern Regional Research Laboratory. Cooking is not employed in making gland-free meals. Numbers 1 to 9 were prepared by the South Texas Cotton Oil Company with the cooperation of the Southern Regional Research Laboratory. Numbers 2 and 9 are hydraulic-press meals and the remaining 7 are screw-press (Hadden et al., '50). Processing conditions were established and chemical properties determined at the Southern Regional Research Laboratory and are given in table 1.

Series 5 consisting of 15 experimental meals was prepared, taking advantage of the information obtained from series 1. Five of these were analyzed in the same manner as was done with series 1. The processing data for these meals are also given in table 1. The standard in this series was an unheated cottonseed meal which had been treated with hexane and ethyl methyl ketone.

TABLE 1

Processing conditions and chemical properties of cottonseed meals

MEAL NO.	MAXIMUM COOKING TIME	MAXIMUM TEMPERATURE	LIPIDS	TOTAL GOSSYPOL	FREE GOSSYPOL-LIKE MATERIAL	MOISTURE	TOTAL [2] NITROGEN	SOLUBLE NITROGEN
	min.	*°F.*	*%*	*%*	*%*	*%*	*%*	*% of total*
				Series 1				
Standard [1]	..	..	less than 1%	..	0.080	8.0	12.0	83.3
1	20	234	3.5	0.63	0.010	4.6	7.56	11.7
3	20	261	3.6	0.50	0.020	4.4	7.69	10.0
4	40	262	3.8	0.51	0.020	4.5	7.73	10.3
5	100	279	5.7	0.14	0.030	3.7	7.04	8.2
6	20	200	3.4	0.43	0.010	4.1	7.10	15.9
7	70	200	3.4	0.43	0.010	4.7	7.25	12.8
8	70	180	3.3	0.40	0.010	4.6	7.46	14.3
2	36	230	5.6	0.70	0.108	7.6	7.40	39.2
9	76	240	5.6	0.41	0.047	6.6	7.66	20.8
				Series 5				
Standard [2]	..	..	0.2	0.115	0.012	10.25	10.38	70.7
1	37	230	3.7	0.658	0.028	6.54	7.80	15.3
9	26	180	6.4	0.566	0.019	6.37	7.52	31.7
10	26	180	6.3	0.613	0.044	6.26	7.25	41.4
13	26	200	5.9	0.590	0.028	5.90	7.04	26.5

[1] Essentially gland-free meal, hexane extracted.
[2] Extracted with methyl-ethyl ketone and hexane.
[3] Moisture and ash free.

The effect of different processing conditions on the protein quality of the meals was evaluated by feeding them at a level of 10% protein (N × 5.3) to weanling male rats from the stock colony of this laboratory.[2] The other ingredients of the ration consisted of salt mixture (Jones and Foster, '42) 4%, corn oil [3] 3%, lard 12%, vitamin A and D concentrate [4] 0.05%, inositol 0.1%, choline chloride 0.2% and sucrose to make 100%. In addition, the following vitamins were added per kilogram ration: thiamine hydrochloride, 5 mg; riboflavin, 10 mg; pyridoxine hydrochloride, 5 mg; nicotinic acid, 5 mg; calcium *d*-pantothenate, 25 mg; *p*-aminobenzoic acid, 300 mg; α-tocopherol acetate, 25 mg; 2-methyl 1,4-napthoquinone, 2 mg; biotin, 100 μg; and folic acid, 2 mg. Some of the animals were fed vitamin B_{12} [5] at a level of 30 or 50 μg/kg. In feeding the meals of series 5, the corn oil was decreased to 2%, the lard to 8% and the sucrose increased correspondingly. Food and water were fed ad libitum, and the weight changes and food intakes were determined weekly. Ten animals were fed each diet, and litter mates were distributed among the various groups in a random fashion. The feeding trials were continued for 6 weeks, and the protein efficiencies (grams gain per gram of protein consumed) of the meals determined.

An acid hydrolysis was made on each sample and the essential amino acids were determined microbiologically using the methods developed in this laboratory (Horn, Jones and Blum, '50). Little information suggesting differences in meals was obtained by assay of the acid hydrolysates. However, vast differences in the nutritive value of these meals were obtained from the animal-feeding experiments. It was believed, therefore, that analysis of an enzyme digest might show why the differences occurred. Since the amount of growth is limited in the microbiological method by the availa-

[2] The strain of rats is the result of mating an albino with a black and white hooded strain. The litters may include white, black and black and white rats.

[3] Mazola.

[4] Squibb's Navitol containing 65,000 USP units of vitamin A and 13,000 USP units of vitamin D per gram.

[5] Squibb's Rubramin or Merck's Cobione.

ble amino acids, the amino acids in the hydrolysates analyzed must be free or in the form of small peptides. Any processing tending to inhibit the digestion of the protein so that the amino acids are no longer freed should then be detected by the microbiological method.

An enzyme system was developed using pepsin, trypsin and hog mucosa for successive 24-hour periods. After this 72-hour digestion period the essential amino acids were determined microbiologically on the solutions. That reproducible results were obtained by this method was shown by the fact that the apparent amino acid availabilities of replicate samples agreed within $\pm$ 8%.

In our earlier work the assay levels of the enzyme digests did not check, varying as much as 50%. Investigation into the matter showed that the buffer in the digests was causing a constant increase in titration for all points on the curve. This was true for all the amino acid standard curves and indicated that the "blank" contained negligible amounts of the amino acid being assayed. When corrections for the titration attributable to the blank were made, the assay levels of enzyme digests agreed well within the experimental error of microbiological assays.

RESULTS

The results of the rat-feeding trials are given in table 2. The results from the microbiological assays of both acid and enzyme hydrolysates are given in table 3. In order to compare the various meals the results from the microbiological assays were calculated to 16% nitrogen and expressed as per cent of the standard. The assays on the acid hydrolysates (table 3) show that except for number 5 there is very little destruction of amino acids by the processing. On number 5, some of the lysine, methionine and histidine appeared to be destroyed by the processing.

Examination of the results on enzyme hydrolysates (table 3) shows that there are differences in the availability of the amino acids in the various meals. Assuming the amount

of the amino acids obtained on the standard (unheated) meal to be the maximum amount freed by the enzyme system used, the heated meals then show differences in digestibility due to the processing. The heat and other processing conditions have caused a change in the protein of the cottonseed so that the enzymes cannot digest it with the same facility as they do the standard meal. All the amino acids appear to be affected. Some amino acids are affected more than others,

TABLE 2

Protein efficiencies of various cottonseed meals

SERIES 1		SERIES 5 [3]	
Meal no.	Protein efficiency	Meal no.	Protein efficiency
1	1.83	1	2.02
2	2.01	9	2.16
3	1.73	10	2.46
4	1.70	10 (no B_{12})	2.28
5	0.52	13	2.31
6	2.26	Standard [4]	2.87
7	2.01		
8	2.16		
9	1.69		
Standard [1]	2.50		
Standard + B_{12} [2]	2.70		

[1] Hexane extracted.
[2] Fifty micrograms per kilogram.
[3] Thirty micrograms B_{12} added per kilogram of ration except where indicated.
[4] Ethyl-methyl ketone and hexane extracted.

depending on the variables of processing. For example, number 4 has less arginine and isoleucine available but much more histidine, lysine and threonine than number 2.

Differences in availability of the various amino acids in the enzyme digests of series 1 may be illustrated as follows: the amounts of amino acids liberated from the standard meal by the enzyme system used when compared with the amounts liberated by the acid hydrolysis of the standard show arginine, lysine and histidine to be 79%, 52% and 33% available. This is the maximum per cent available by this system. Any

TABLE 3

Effect of processing on content and availability of essential amino acids of cottonseed meals[1]

	ARGININE		HISTIDINE		ISOLEUCINE		LEUCINE		LYSINE	
	A	E	A	E	A	E	A	E	A	E
Std.	100 (12.37)[2]	100 (9.77)	100 (2.86)	100 (0.93)	100 (3.53)	100 (2.17)	100 (6.08)	100 (3.85)	100 (4.93)	100 (2.54)
1	96.4	83.8	96.2	79.6	92.6	98.6	99.0	94.3	100	48.0
2	97.0	93.6	92.7	53.7	103.1	97.2	103.1	87.0	96.6	43.3
3	94.9	84.8	95.1	58.1	98.0	74.6	95.0	76.6	95.3	67.7
4	96.0	80.4	90.9	69.9	102.0	76.9	100.0	79.2	96.4	65.7
5	94.6	69.1	87.9	40.8	94.0	86.1	94.0	77.1	80.7	31.9
6	100.6	88.0	102.8	77.4	99.7	100.0	95.2	101.3	103.4	71.7
7	100.6	88.1	90.9	88.2	103.1	100.0	106.1	89.3	99.4	77.2
8	97.8	88.1	102.8	77.4	100.9	100.5	96.8	99.2	98.2	74.0
9	99.0	89.2	93.7	95.4	101.9	77.9	96.7	102.3	97.4	34.6
Std.	93.1	86.6	96.2	95.4	106.5	96.7	100.0	94.5	96.1	93.7
1	96.0	73.5	86.0	64.5	98.3	97.7	98.7	91.2	92.9	59.8
9	99.8	70.2	85.3	83.9	107.3	91.2	97.5	100.5	98.4	58.3
10	95.1	80.1	90.5	84.8	108.2	103.2	99.6	91.4	97.4	60.2
13	100.3	74.8	91.6	81.7	115.0	100.0	100.6	94.6	89.0	59.8
Comm'l.	92.3	70.8	86.1	65.6	104.0	97.2	96.7	82.1	89.9	62.6
Av.	96.9		93.2		102.3		98.6		95.4	

	METHIONINE		PHENYLALANINE		THREONINE		TRYPTOPHAN		VALINE	
	A	E	A	E	A	E	A	E	A	E
Std.	100 (1.50)	100 (1.12)	100 (5.41)	100 (4.31)	100 (3.69)	100 (2.48)		100 (0.78)	100 (4.98)	100 (2.90)
1	92.0	50.9	93.9	79.8	107.7	60.1		79.5	102.0	77.6
2	86.7	44.6	102.0	83.8	114.1	57.7		71.8	104.2	70.0
3	93.3	50.0	92.2	81.4	99.5	60.1		80.8	101.6	73.8
4	90.0	51.8	94.8	76.9	102.4	82.3		85.9	102.0	79.0
5	81.3	35.7	88.4	81.2	107.3	46.8		50.0	101.6	71.0
6	102.0	49.1	90.2	89.3	105.2	64.9		80.8	103.0	92.8
7	94.0	57.1	94.6	86.1	96.5	60.9		76.9	102.0	101.4
8	113.3	59.8	91.5	86.5	103.5	100.0		80.8	100.0	103.5
9	104.0	44.6	90.4	76.8	95.7	81.1		66.7	101.6	94.8
Std.	104.0	76.8	97.6	103.3	101.4	78.6		99.7	107.2	96.9
1	82.0	42.9	93.2	77.3	96.8	79.4		62.8	104.4	102.1
9	86.7	46.4	96.5	80.3	103.0	90.3		70.5	106.4	100.3
10	82.0	50.9	94.3	84.9	113.3	98.0		70.5	110.4	113.8
13	90.7	46.4	93.2	83.5	117.3	80.7		68.0	102.4	101.7
Comm'l.	82.0	48.2	97.0	86.8	104.6	64.1		69.2	100.0	91.4
Av.	92.9		94.0		104.5				103.3	

[1] Expressed as per cent of unprocessed meal.

[2] Actual amino acid content of standard calculated to 16% nitrogen.

deviations from these availabilities reflect changes due to processing. In series 5 it can be seen that, with the exception of the commercial meal, histidine, lysine, arginine, tryptophan, and methionine were less available in the heated than in the unheated meal; leucine, isoleucine, and valine were as available as in the standard.

Correlation of microbiological and animal feeding results

In order to evaluate the results obtained from the amino acid assays on both series, a "Nutritive Index" was devised as follows: the amount of the 10 amino acids set free by the enzyme system for the standard of series 1 was taken as 100%. The values obtained for the other meals were divided by the values obtained for the standard of series 1. This was done for each essential amino acid, giving a series of percentages for each meal. These, when added together and divided by 10, gave the Nutritive Index for that meal or average availability to the microorganisms of the 10 essential amino acids. For example, the enzyme digest of the standard for series 1 when assayed for lysine gave 2.54%; the assay for meal number 1 gave 1.22%; therefore the fraction for lysine for meal number 1 was 0.48, or 48%. When this was done for all 10 essential amino acids for meal number 1, and the percentages added and divided by 10, the Nutritive Index for that meal was found to be 75.2% compared with 100% for the standard. The Index values can be obtained directly from table 3.

In the rat-feeding trials of series 1 vitamin B_{12} was included in the ration in only one test. In series 5, vitamin B_{12} was added in the testing of each meal, but one meal was also fed without B_{12}. In series 1 the values for protein efficiencies for the meal fed with and without B_{12} were 2.70 and 2.50. In series 5 the values were 2.46 and 2.28. These values represent an increase of 8.00 and 7.9% in the protein efficiency due to the presence of the vitamin. In the microbiological assays the Nutritive Indices of both series are calculated

with the standard of series 1 as 100. In order to compare the rat-growth studies from the two series we have decreased the values for series 5 by 8%, the amount by which the nutritive value was increased by the presence of B_{12}. In order to compare the results of the rat-feeding trials and the amino acid assays, the results of the rat-feeding trials are expressed as per cent of the protein efficiency of the standard meal of series 1.

TABLE 4

Nutritive indices calculated from microbiological data compared with indices from rat-growth studies

NUMBER COTTONSEED MEAL	NUTRITIVE INDICES	
	From microbiological studies	From rat growth studies
	Series 1	
Standard	100	100
8	87	86
7	83	80
6	82	90
9	76	68
1	75	73
4	75	68
3	71	69
2	70	80
5	59	22
	Series 5	
Standard	92	106
10	84	90
9	80	80
13	79	85
1	75	74

In table 4 the Nutritive Indices obtained from the microbiological assays are compared with the Nutritive Indices obtained by rat feeding. In considering the relative order of nutritive value there is good correlation between the Indices.

It is believed that with a little more experience and refinement of the technique the enzyme microbiological method will prove to be a useful tool in evaluating protein foods with fair reliability.

It might be pointed out that attempts to correlate the feeding and microbiological methods by using one, two or even three of the most labile amino acids met with no success. It appears that consideration of all the essential amino acids must be given to evaluate the meal.

The results on chick feeding obtained by Milligan and Bird ('51) on the meals of series 1 agree favorably with our results.

SUMMARY

It has been demonstrated that various methods of processing cottonseed meals alter the nutritional value of the protein.

Two methods of evaluating these meals were used; the rat assay method, which showed that the nutritional value of these meals for the rat was considerably altered by the processing, and the microbiological method, which showed that the changes in nutritional value were due to a change in the availability of the amino acids. Different methods of processing affected the availability of different amino acids. The assay of acid hydrolysates showed that only very severe temperatures and pressures caused any destruction of the amino acids. Comparison of results from assays of acid and enzyme hydrolysates by the microbiological method showed that, although the amino acids were present, some of them were bound in such a way that they were no longer available to the microorganism.

An enzyme digestion system has been worked out which appears to give good reproducibility of results; the availability of the digestion products to the microorganisms correlated well with the results of rat feeding.

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THE EFFECT OF DIETARY FAT AND CALORIC RESTRICTION ON PROTEIN UTILIZATION [1]

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FOUR FIGURES

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The experiments of Willman et al. ('47) and Swanson ('51) indicate that fats have a greater nitrogen sparing effect than carbohydrates in rats fed a low caloric diet. Similar results have been reported in man by Schwimmer and McGavack ('48). On the other hand, Allison et al. ('46) reported data on the dog that suggested little if any difference in the protein sparing action of fat or carbohydrate.

The present experiments were undertaken to investigate more fully the role of dietary fat with concomitant caloric restriction on the utilization of dietary protein in the dog. Since the incorporation of large amounts of fat into the diet might cause intestinal disturbances, digestibility studies were completed in normal and protein-depleted dogs.

METHODS

Adult dogs were fed the purified diet used in previous studies by Allison and Anderson ('45) as modified by Rosen-

[1] These studies were supported by the Protein Metabolism Fund of the Bureau of Biological Research, Rutgers University.

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The data reported in this paper were submitted to the Graduate Faculty, Rutgers University, in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

thal and Allison ('51). Since the caloric requirement of dogs varies somewhat with the size and breed, the results are expressed in terms of the average per cent of the calories required to maintain the body weight of adult dogs, as determined in this laboratory. Protein (Labco Casein) was incorporated in the diet and the animals were fed on the basis of grams protein nitrogen/day/kilogram probable body weight (Cowgill, '28). The calorie, fat (lard) and protein content of the diet was increased or decreased by substitution of carbohydrate.

Nitrogen balance indexes were determined by methods previously developed in this laboratory (Allison and Anderson, '45). The nitrogen balance index (K) is a function of the amount of nitrogen retained in the body of the animal and is defined as the rate of change of nitrogen balance with respect to absorbed nitrogen. The slope of the line then becomes the nitrogen balance index. This equation is constant in the region of negative or low positive nitrogen balance, but it becomes a decreasing variable in the region of high positive nitrogen balance. To determine the index, the dogs were placed on a protein-free diet for 7 days, followed by 8 days of protein feeding (two periods), and again followed by 5 days of protein-free feeding. Appropriate collections of urine and feces (with carmine markers) were made and analyzed for nitrogen by the Pregl micro-Kjeldahl method.

The digestibility of lard was determined by placing dogs on the diet containing egg albumin as the protein source. They received 0.12 gm nitrogen per day per kilogram of body weight and 100% of their caloric requirement. The fat in the food and feces was determined as the weight of free fatty acids by a modification of the wet extraction method described by Fowweather ('26). Plasma proteins were determined by the salt fractionation method of Howe ('21). After the normal data were obtained, the dogs were depleted by feeding a protein-free diet for 30 days, and the experiments were repeated on the depleted animals.

RESULTS

Allison et al. ('46) have shown that the nitrogen balance index equation becomes curvilinear in the region of positive balance. They also demonstrated that dogs fed restricted diets could not maintain positive nitrogen balance. The data presented in figure 1 show the effect of dietary protein on

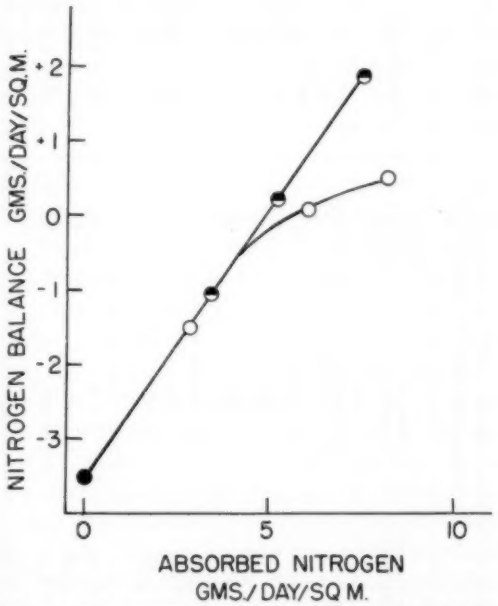

Fig. 1 Nitrogen balance versus absorbed nitrogen for dogs fed various protein intakes. The half black circles (◒) represent dogs fed 100% of the caloric requirement and the open circles (○) represent dogs fed 25% of the caloric requirement. The solid circle (●) represents nitrogen excretion on a protein-free diet.

nitrogen balance at different caloric intakes. These data were obtained by using diets limited to a fat content of 5% of the calories. All of the experimentally determined values have been adjusted to the same NE_o value in accordance with the equation $NB = K(AN) - NE_o$, where NB is nitrogen balance, K is the nitrogen balance index, AN is absorbed nitrogen and

TABLE 1

The effect of dietary fat on the nitrogen balance index of casein obtained on dogs fed 0.15–0.20 gm casein nitrogen/day/kilogram body weight

FAT CONTENT OF DIET	NO. OF DETERMINATIONS	ABSORBED NITROGEN	NITROGEN BALANCE	PROTEIN-FREE NITROGEN BALANCE	DIGESTIBILITY [1]	NITROGEN BALANCE INDEX
% Cal.		*gm/day/m²*	*gm/day/m²*	*gm/day/m²*	*%*	
			100% Caloric requirement			
5	14	3.23	+ 0.19	— 2.17	100 ± 0.46 [2]	0.74 ± 0.01
30	10	3.33	— 0.27	— 2.64	98 ± 0.95	0.72 ± 0.03
60	10	3.44	— 0.01	— 2.39	98 ± 0.67	0.71 ± 0.05
85	10	3.38	— 0.29	— 2.69	97 ± 0.22	0.71 ± 0.03
			50% Caloric requirement			
5	12	2.58	— 1.53	— 3.31	99 ± 0.29	0.70 ± 0.05
85	6	2.51	— 0.79	— 2.51	97 ± 2.0	0.69 ± 0.02
			25% Caloric requirement			
5	6	2.90	— 1.39	— 3.38	99 ± 1.0	0.70 ± 0.06
50	8	2.67	— 0.70	— 2.50	100 ± 1.0	0.64 ± 0.01
85	6	3.01	— 2.08	— 3.31	98 ± 2.2	0.41 ± 0.02

[1] True digestibility corrected for endogenous fecal nitrogen as determined by feeding a protein-free diet.

[2] Standard error $= \sqrt{\frac{\Sigma d^2}{n\,(n-1)}}$. This equation refers to all such data presented in this paper.

NE_o is the excretion of nitrogen on a protein-free diet. It is apparent that dogs fed restricted diets utilize nitrogen in a normal fashion when the protein content is relatively low. Additional dietary protein results in a small positive balance which cannot be increased further no matter how much protein is included in the diet. Thus, the reduction of the nitrogen balance index indicates the utilization of dietary protein to supply needed calories.

The data of table 1 demonstrate that the nitrogen balance index of casein is not altered from normal by varying the fat content of the diet from 5 to 85% of the total calories when the caloric intake is 100 or 50% of the calories required to maintain body weight. However, when the intake was reduced to 25%, an increase of dietary fat resulted in decreased nitrogen balance indexes below normal values.

The data of table 2 illustrate the effect of dietary fat on the nitrogen balance obtained in dogs fed large amounts of casein and restricted caloric intakes. In dogs fed 100% of the daily caloric requirement, nitrogen balance is not altered from normal by varying the fat content of the diet. At 25%, nitrogen balance is reduced slightly as is to be expected from the data shown in figure 1. The reduction of dietary calories results in a greater excretion of nitrogen in animals fed a protein-free diet, but the incorporation of large amounts of fat in the diet has no further effect. The nitrogen balance indexes were not calculated for the data obtained in dogs fed diets restricted in calories, due to the curvilinear nature of the nitrogen balance index equation. When sufficient calories are given, increasing the fat content of the diet does not alter the index.

The recent report of Swanson ('51) indicates that in rats previously depleted of protein, the removal of fat from restricted diets leads to an increased excretion of nitrogen. The protein sparing effect of fat may, however, vary with the physiological state of the animal. Protein depletion, for example, results in an imbalance of tissue proteins and enzyme systems which can alter the physiology of the animal (Alli-

TABLE 2

The effect of dietary fat on the nitrogen balance index of casein obtained on dogs fed 0.30 gm casein nitrogen/day/kilogram body weight

FAT CONTENT OF DIET	NO. OF DETERMINATIONS	ABSORBED NITROGEN	NITROGEN BALANCE	PROTEIN-FREE NITROGEN BALANCE	DIGESTIBILITY [1]	NITROGEN BALANCE INDEX
% Cal.		*gm/day/m²*	*gm/day/m²*	*gm/day/m²*	*%*	
			100% Caloric requirement			
5	8	5.16	+ 1.91	— 1.97	98 ± 1.1 [2]	.75 ± .02 [2]
63	7	4.95	+ 1.46	— 1.82	98 ± 0.91	.66 ± .03
85	7	5.11	+ 1.93	— 1.61	97 ± 0.90	.69 ± .02
			25% Caloric requirement			
5	6	6.05	+ 0.80	— 2.76	99 ± 0.61	...
63	6	6.02	+ 0.32	— 2.94	98 ± 0.57	...

[1] True digestibility corrected for endogenous fecal nitrogen as determined by feeding a protein-free diet.
[2] Standard error.

son, '51). The experiments reported here were performed with well nourished animals, and it is conceivable that different mechanisms may be operative in these animals as contrasted with those depleted of protein. These and other dietary factors which may affect the preservation of body tissues require further study.

Experiments by Munk and Rosenheim (Van Noorden, '07) demonstrated that the digestibility of fats was greatly reduced when dogs were fed low-protein diets for 6 to 8 weeks. Post mortem examinations revealed severe lesions of the gastrointestinal tract which might account for the loss of digestive powers. On the other hand, Coffey et al. ('40) found only slight variations in the digestibility of fats in dogs, and Barnes et al. ('44) found in rats that readily digestible foods such as lard are not affected in digestibility by the protein content of the diet. In the experiments reported here, the digestibility of protein is not altered from normal under any of the experimental conditions as shown in tables 1 and 2. The digestibility of fat, presented in table 3, is also normal in dogs with adequate protein stores. However, in dogs depleted of protein, fat digestibility is reduced from 98% to 88%. In no instance was any evidence of gastrointestinal disturbances observed with the high-fat diets.

Rosenthal and Allison ('51) have recently demonstrated the effects of caloric restriction on nitrogen retention. Two responses to a restricted caloric intake were recognized. The first response was an increase in the excretion of urinary nitrogen without any change in the nitrogen balance index of the dietary protein. The second response was a decrease in the nitrogen balance index reflecting a shift in the mechanism of nitrogen metabolism. They also indicated that resistance to caloric restriction was correlated, in part, with the magnitude of the protein stores of the body. As pointed out by Elman ('37), the body tissues may furnish a good deal of the daily caloric needs when the diet is restricted. Further studies to be described in this report indicate that

TABLE 3

Average digestibility of fat (lard) in normal and protein depleted dogs

NO. OF DETERMINATIONS	ALBUMIN / GLOBULIN	FAT CONTENT OF DIET	FATTY ACID INTAKE	FATTY ACID EXCRETED	DIGESTIBILITY [1]
		% Cal.	*gm/m²/day*	*gm/m²/day*	*%*
			Normal		
5	1.39 ± 0.11 [2]	0	0 ± 0 [2]	0.79 ± 0.02 [2]	...
		30	39 ± 2	1.21 ± 0.26	99 ± 0.01
		60	79 ± 3	2.66 ± 0.15	98 ± 0.01
		85	115 ± 6	2.77 ± 0.39	98 ± 0.01
			Depleted		
4	0.71 ± 0.03	0	0 ± 0	1.11 ± 0.6	...
		30	40 ± 3	8.12 ± 2.6	82 ± 2.9
		60	82 ± 5	11.2 ± 2.3	88 ± 2.1
		85	118 ± 5	14.7 ± 2.9	88 ± 1.5

[1] True digestibility corrected for endogenous fecal fat as determined by feeding a fat-free diet.
[2] Standard error.

the magnitude of the caloric reserves (body fat) also plays a role in the resistance of dogs to caloric restriction.

The data in figure 2 were obtained on two dogs fed a protein-free diet (white bars) alternating with protein feeding (slanted bars). The alternate feeding of protein-free and protein diets was done to make the calculation of nitrogen balance indexes possible, and to study, through protein-free

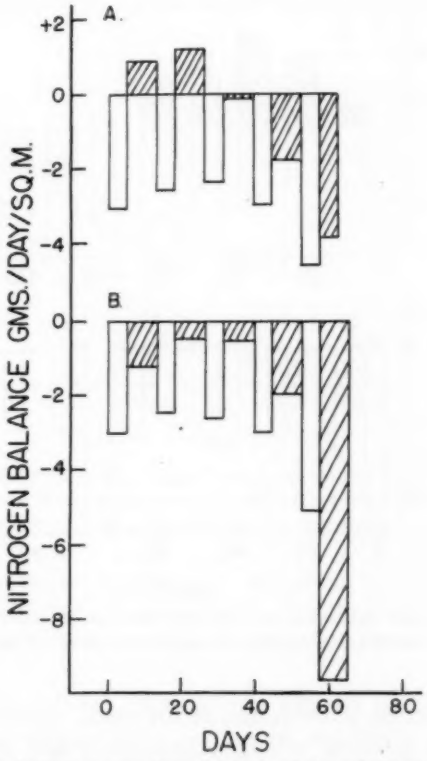

Fig. 2 Nitrogen balance versus days fed 25% of the caloric requirement. The white bars represent protein-free feeding periods and the slanted bars represent protein periods. Dog A was fed 0.30 gm casein nitrogen per day per kilogram of body weight and Dog B was fed 0.15 gm casein nitrogen per day per kilogram body weight.

feeding, the effects of caloric restriction on body nitrogen. The caloric intake was limited to 25% of the daily requirement and the fat content of the diet was maintained at 5% of the calories. Dog A was fed 0.30 gm casein nitrogen per day per kilogram of body weight, an amount sufficient to place the animal in positive balance. Dog B was fed 0.15 gm casein nitrogen per day per kilogram, which was not sufficient to maintain nitrogen equilibrium.

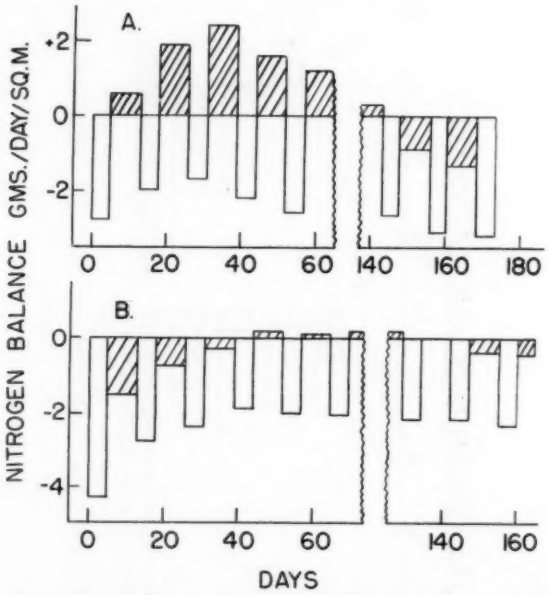

Fig. 3 Nitrogen balance versus days fed 25% of the caloric requirement for two dogs very resistant to the effects of caloric restriction. The data are expressed as in figure 2.

The reduction in excretion of nitrogen, shown by a gradual increase in positive nitrogen balance, could be expected as a result of the depleting effects of periods in negative nitrogen balance plus the effects of restricted caloric intake. The depletion phase was followed by an interval in which the excretion of nitrogen reached high values, and the animals

became quite ill. The data in figure 3 pertain to two dogs which were very resistant to the depleting effects of a caloric restriction, and therefore required many days to reach a state of negative nitrogen balance. These data have been selected to demonstrate the extreme responses of animals to partial starvation.

The effect of feeding restricted diets on the body weight of dogs is illustrated in figure 4. The ratio of the actual weight to the probable weight makes possible a common basis

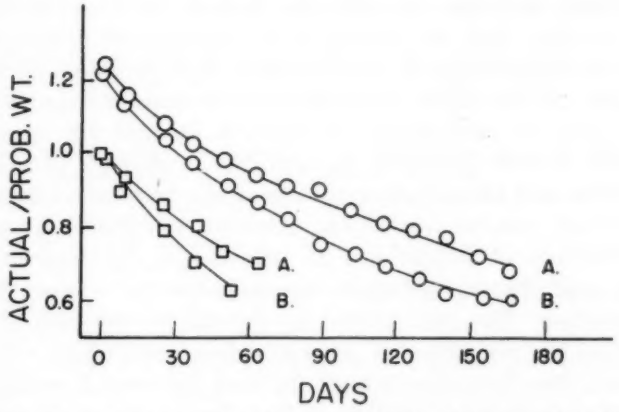

Fig. 4 The ratio of the actual weight to probable weight versus days fed 25% of the caloric requirement. The squares (□) represent the weight loss in the dogs shown in figure 2, and the circles (○) represent the dogs shown in figure 3.

of comparison between animals. Although the rate of loss in weight is similar in all animals, the heavier dogs have more reserves, and it requires a longer period of time to deplete them.

The nitrogen balance index is not altered while the tissue reserves are adequate but is reduced markedly during the shift toward negative nitrogen balance (Rosenthal and Allison, '51). The shift toward negative balance has been interpreted as one of utilization of both body and dietary nitrogen for energy purposes, leading to marked tissue breakdown.

SUMMARY

These experiments demonstrate that the incorporation of large amounts of fat in the diet does not alter the utilization of dietary protein when the caloric intake is at an optimal level. Nitrogen utilization may be reduced, however, when large amounts of fat are fed in conjunction with diets restricted in calories and protein. The addition of large amounts of protein to calorically deficient diets results in the use of some of the protein for energy purposes but prevents an increased excretion of nitrogen caused by high-fat diets. The average dog can digest large amounts of protein and fat, but digestibility of fat decreases with depletion of body protein. When dogs are subjected to caloric deprivation, they may be maintained in positive balance for variable lengths of time provided the quantity of dietary protein is sufficient and the caloric reserves of the body are adequate. Continued caloric restriction, however, eventually leads to marked loss of weight and an increase in the excretion of body and dietary nitrogen associated with severe tissue destruction. The role played by the caloric reserves of the body on the resistance of dogs to caloric restriction are discussed. The data also indicate the need for careful evaluation of nitrogen balance studies so that the variables of physiological state and composition of the diet are comparable.

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The author wishes to express his appreciation to Dr. James B. Allison, Rutgers University, for his encouragement, guidance and advice during the course of this investigation.

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BASAL HEAT PRODUCTION AND ELIMINATION OF THIRTEEN NORMAL WOMEN AT TEMPERATURES FROM 22°C. TO 35°C. [1]

EUGENE F. DuBOIS, FRANKLIN G. EBAUGH, JR., AND JAMES D. HARDY
WITH THE TECHNICAL ASSISTANCE OF G. F. SODERSTROM [2] AND E. IRENE STEVENS
The Russell Sage Institute of Pathology in affiliation with the New York Hospital and Department of Physiology, Cornell University Medical College, New York City

SIXTEEN FIGURES

(Received for publication July 22, 1952)

It is well known that women have much more influence than men when it comes to adjusting room temperatures. That is why it seems strange that they have been neglected in studies of heat loss and the mechanism of temperature regulation. Many investigators have studied men, few have included women.

It is this neglect of the better half of the population that started our calorimeter studies of normal women in 1938, continued with war interruptions until 1948. Soon the reasons for the previous avoidance of women became apparent. It is difficult to find busy young women who can interrupt their work for a long series of tests extending over

[1] Clinical Calorimetry Paper, no. 55.

[2] G. F. Soderstrom died in October, 1948, at the age of 73. His services to calorimetry had been noteworthy. He built the Sage respiration calorimeter in 1912 and for 35 years operated the control table. He devised and constructed many new pieces of apparatus, these, like his techniques, always a little better than necessary. He took an active part in all experiments and he was the joint author of many papers.

a period of several months. Many young women, otherwise perfectly normal, have irregularities in the time of menstruation and occasional anovulatory cycles. Also, women show rather large individual differences, and it happened that the group of 6 normal women studied before the war had, on the average, a greater response to cold than the 7 women studied in the second series after the war. Since a much better picture is given by a group of 13 than by 6 or 7, the figures for all 13 women are assembled in this paper, even though some of the data have been published in previous reports (Hardy and Milhorat, '39; Hardy and DuBois, '40; Hardy, Milhorat and DuBois, '41; Hardy, Shorr and DuBois, '47; Hardy, Ebaugh, Stevens and DuBois, '50). The technique in the two series was identical, and it has been described in papers 49 to 53 of this series (Clinical Calorimetry, '38). The women came into the laboratory at about 8:30 A.M. A nurse helped them undress at about 9, weighed them and then put them in the darkened Sage Calorimeter. The box was sealed and after a preliminary period of about three-fourths of an hour the first experimental period ("First Hour") was started. Most of the experimental periods began between 10:15 and 11 A.M. During the preliminary period the women measured the skin temperatures in 20 places using the Hardy radiometer. This only required two or three minutes and involved the expenditure of one or two cal. Immediately after the end of the experiment they measured the surface temperature again. In many experiments, as is shown in the figures, skin temperatures were measured at the beginning of the second and subsequent periods. For this reason the estimates of the basal rates in table 1 are based on the data for the first hour only.

The observations at different calorimeter temperatures were run in random order, but as a rule the first was in or near the comfort zone. Since the tests had to be planned a week or two in advance it was impractical to limit them to any one phase of the menstrual cycle.

The women were instructed to observe and report any discomfort, sensations of cold or warmth, tensing of the muscles and the exact time of onset of shivering. At the end of the experiment they recorded their sensations in detail. All were cooperative but a few could not complete the series in the cold on account of changes in work schedules. In most experiments there were two periods of one hour each. Sometimes periods longer or shorter than an hour were used on account of technical difficulties or approaching chills. In some cases there was only one basal hour followed by factors such as alcohol or cold water, the effects of which will be reported in subsequent publications. Some of the best subjects could stand experiments lasting from three to 5 hours.

With well-trained subjects the basal figures obtained in the Sage respiration calorimeter are practically identical with those found using the Benedict type of closed circuit apparatus in 6-minute periods. Subjects not so well-trained find it difficult to remain motionless two or three hours and the metabolism tends to rise, perhaps on account of fatigue or slight restlessness. Several experiments have been excluded from this series because it was noted at the time of the experiment that the conditions were not "standard" or basal. The women in the calorimeter could be watched; movements were detected promptly by a slight rise in air temperature and sudden elevation of the small spirometer on the top of the calorimeter. A work adder was attached to this spirometer giving a record of all movements during each hour. In previous years it had been shown that this was delicate enough to record the movement of a man when he twisted his mustache.

EXPERIMENTAL

Subjects

Table 1 shows the data for the subjects. All were given careful physical examinations and every individual was found to be in good health. The records of the women provide more details. The experiments are listed with their dates in the order of rising temperatures to make easier comparison with the graphs.

Subjects 1, 2, 4 a, 5, 6 and 7 have the same numbers as in our previous reports. Graphs of subjects 2 and 4 a (Hardy and DuBois, '40) and data of subjects 1 and 2 with averages of subjects 1 to 7 (Hardy, Milhorat and DuBois, '41) have been published. These data are now recalculated in slightly altered form with more emphasis on the differences between the first and second experimental hours.

Number 3, J. W., of the first series has been omitted from this report on account of her amenorrhea. The 1940 series of A. S. (4 b) is published for the first time.

Number 1, H. G., is a research associate who has been the subject of many kinds of experiments. Calorimeter temperature was 23.1 on February 2, 1938, 15 days after menses. She had taken a small early breakfast of the type that causes little or no increase of metabolism. First hour cold and tense, second

TABLE 1

Normal women subjects

NO.	INITIALS	AGE	AV. WT.	HEIGHT	SURFACE AREA	CAL. SQ. M/HOUR COMF. ZONE FIRST HR.	NO. OF EXP.	
							Comfort zone	Total
			kg	*cm*	*sq. m*			
1	H.G.	35	64	175	1.77	32.4	2	6
2	P.N.	25	60	162	1.62	30.7	5	10
4a	A.S. 1939	21	76	162	1.82	28.8	5	13
4b	A.S. 1940	22	64	162	1.69	28.1	2	8
5	H.E.	23	56	168	1.63	32.3	2	7
6	E.M.	42	53	169	1.61	33.1	1	3
7	A.B.	26	54	165	1.59	30.2	3	3
8	M.S.	24	61	175	1.75	34.1	3	3
9	B.R.	21	62	161	1.65	30.6	3	6
10	G.C.	28	78	176	1.93	27.6	2	4
11	P.H.	22	55	163	1.58	32.1	2	4
12	H.H.	24	40	154	1.34	34.5	1	1
13	L.T.	23	51	163	1.53	33.5	3	4
14	R.C.	23	76	172	1.88	29.3	3	4

period slight shivering. Temperature was 23.3 on February 9, 1938, 22 days after menses. Temperature was 25.2 on January 19, 1938, one day after menses. In the second and third hours she was cold and had a desire to urinate. In the third period there was slight shivering. Temperature was 26.7 on January 12, three days before start of menses. Temperature was 30.3 on March 11, 1938; second day of menses, no discomfort. Temperature was 33.7 on March 2, 1938, 8 days before menses, had a small early breakfast. First hour warm and drowsy; second hour, a slight headache.

Number 2, P. N., is an artist's model, and is married. Menstrual data not available. Calorimeter temperature was 24.0 on March 25, 1938. Period terminated 20 minutes after start on account of an approaching chill. Tempera-

ture was 24.9 on January 21, 1938. In the third period there was a mild chill. Temperature was 26.8 on January 14, 1938. Temperature was 28.3 on February 16, 1938. Temperature was 29.8 on February 18, 1938. Temperature was 31.2 on March 18, 1938. Temperature was 32.0 on February 25, 1938. Temperature was 35.5 on March 4, 1938. By 11: 30 she was sweating all over the body. Temperature was 35.8 on March 8, 1938. Slept for a few minutes in first period. Sweating profusely and slightly restless. Felt cooler towards end of the experiment.

Number 4 a, A. S., a research associate who has served as the subject of many experiments. She could remain quiet in the calorimeter for long periods. During the first year of experiments in 1939, her weight varied between 75.2 and 76.5 kg. During the summer she decided that she was too heavy and reduced her diet until she had lost about 12 kg. On December 7, 1939, when the second series (unpublished in our first reports) started, she weighed 63.8 kg. She maintained an uncomfortably low diet and when the experiments ended in May, 1940 she weighed 64.5 kg. Her menstrual intervals were about 28 days. Her data for 1939 and 1940 are given below.

Nineteen thirty-nine series. Calorimeter temperature was 22.4 on May 16, 4 days after menses; she was cold and on the verge of shivering during the last few minutes. Had a chill on coming out of the calorimeter. Temperature was 22.6 on April 6, 18 days after menses. Temperature was 23.5 on May 23, 11 days after menses. Temperature was 24.2 on March 9, 18 days after menses. Temperature was 24.9 on May 18, 6 days after experiment. Doubtful slight shivering in second hour. Temperature was 27.9 on March 10, 19 days after menses. Temperature was 30.0 on May 9, two days before menses. Temperature was 31.0 on May 24, 12 days after menses. Sweating in axilla and palms. Temperature was 31.7 on March 23, 5 days after menses. Temperature was 33.1 on March 30, 12 days after menses. Temperature was 34.9 on April 20, 5 days after menses.

Nineteen forty series. Number 4 b, A. S. Data on menstrual periods lacking. Calorimeter temperature was 22.1 on February 8. Tense during last half hour. Chilled slightly on coming out of calorimeter. Temperature was 22.2 on February 14; humidity 70%. This experiment and those at 22.3, on February 6, and 22.4 on January 23, were not used in averaging percentage losses by radiation convection and vaporization because the humidity was high in all three experiments. In the first two, the calorimeter felt stuffy. At 22.4, with the humidity 55% to 59%, she did not complain of stuffiness. Temperature was 23.9 on December 12, 1939. Temperature was 27.8 on January 31, 1940; humidity 50% to 57%; comfortable. Temperature was 27.9 on March 10. Temperature was 33.3 on May 22.

Number 5, H. E., is a housewife. This young woman had been examined the previous year in the dispensary on account of loss of weight and anemia. Her thyroid was slightly enlarged. In 1939 she was in good health. There was no evidence of constitutional disease and her weight loss was ascribed to frequent colds and long hours of work. The menstrual intervals were 29 to 30 days. Calorimeter temperature was 22.6 on March 16. Chilly, relaxed, and no shivering. Menses started April 9. Temperature was 24.3 on March 21. Temperature was

26.0 on April 18. Temperature was 27.5 on March 28. Temperature was 29.9 on March 17. Temperature was 32.0 on February 17. She was "hot but not uncomfortable," and perspired a little. Temperature was 33.9 on April 4. First hour warm, but not uncomfortable. Second hour, hot, uncomfortable, and sweating profusely.

Number 6, E. M., is a professor of physiology, who has lived most of her adult life in India, and has been the subject of many experiments. Her basal metabolic rate in Madras averaged 28.5 cal. per square meter per hour. Recently in New York it was reported as 32.2. Her hemoglobin was 65%. She has always been susceptible to cold. Calorimeter temperature was 24.7 on February 20, 1940. Feet cold, and slight twitching of muscles. She had mild shivering after she came out of the calorimeter. Temperature was 26.4 on February 21. Chilly, and slight twitches of muscles; goose flesh. She shivered when she came out of calorimeter. Temperature was 32.4 on February 16. Comfortable.

Number 7, A. B., is a housewife. Calorimeter temperature was 28.3 on April 12, 1938, one day before menses. Temperature was 30.0 on April 19, 4 days after menses. In these two experiments the skin temperature measurements were unreliable so that it was impossible to measure radiation and convection. Temperature was 31.9 on April 21. Temperature was 32.0 on May 10, second day of menses. In all of these experiments she was comfortable and quiet.

Number 8, M. S., is a technician. Her general health was excellent, but she was losing weight. At the end of the experiments a careful study in the clinic was negative. Her menses were regular with little discomfort. Calorimeter temperature was 28.1 on January 19, 1939, 8 days before menses. First hour warm and comfortable, second hour slightly cold; quiet. Temperature was 30.1 on January 26, day before menses. Temperature was 30.2, last day of menses. No discomfort.

Number 9, B. R., is a student nurse. She was in good health but her weight was fluctuating as a result of dieting. Her menstrual interval was 21–28 days. Vaginal smears in June, 1948, showed an anovulatory cycle with poor follicular activity. Maximal cornification was 1%. Most smears showed predominantly large nucleated superficial cells and cytolysis. Calorimeter temperature was 22.2 on May 15, 1947, 61.7 kg, 7 days after menses. During the last 10 minutes of the experiment she was tense. Five minutes after the end of the experiment she shivered all over her body. Temperature was 24.0 on June 19, 60.0 kg, last day of menses. Temperature was 26.1 on May 8, 61.4 kg, third day of menses. Temperature was 30.0 on May 13, 61.2 kg, 5 days after menses; a little too warm, and slight sweating. Temperature was 31.7 on May 22, 62.5 kg; 14 days after menses; a little too warm and slight sweating.

Number 10, G. C., is a dietitian who has lived in the South until she came to New York 5 months ago. She "feels the cold" more than her companions. Up to 9 months previous to the experiments she had been given thyroid intermittently on account of overweight and one low BMR. At the time of the experiments there was no evidence of hypothyroidism except for a low basal. Her menses which are normal began on March 6 and on April 4, 1948. Vaginal smears showed ovulation between April 16 and 18. Calorimeter temperature

was 24.4 on March 31, 1948. Cold and tense, but not shivering. Temperature was 26.8 on March 24; felt slightly cool the first hour. Temperature was 30.0 on March 23; slightly cool. Temperature was 30.7, and she felt very comfortable.

Number 11, P. H., is a nurse. Weight stable. Menstrual periods began March 29 and April 25, 1948. The vaginal smears during the experimental period showed an anovulatory cycle with good follicular activity reaching maximal cornification at 60% on April 16. Calorimeter temperature was 24.9 on April 15, 1948. Cold but relaxed. Temperature was 30.1 on April 13. Felt comfortable. Temperature was 31.3 on April 7. Uncomfortably warm, but relaxed with slight sweating in axillae.

Number 12, H. H., is a nurse of Polish descent, petite, weight stable. In 1947 she had dysmenorrhea for three months, and she was studied in the Endocrine Clinic through two ovulatory cycles. The vaginal smears showed maximal cornification of 40% and 25%. The luteal phase was normal. In 1948, at the time of the experiments, the menses were regular with a 32-day cycle. A menstrual period began March 26. Temperature was 25.9 in April, 1948; felt cool. The experiments were terminated by the news of the death of her brother.

Number 13, L. T., is a nurse. Normal menstruation history, menses began on April 22 and on May 24, 1948. The May 25–June 25 cycle was followed by vaginal smears and morning basal temperature. Ovulation probably occurred June 12–13 with maximal cornification on June 12. The temperature curve began to rise June 12. Calorimeter temperature was 24.0 on May 18, 1948; cool, no inclination to shiver. Temperature was 26.2 on May 13. Hands and feet cold, otherwise comfortable. Temperature was 28.2 on May 11; comfortable. Temperature was 31 on May 20.

Number 14, R. C., is a nurse. She was somewhat overweight and her weight remained constant during the last year. Four years ago she had received thyroid therapy but there is no evidence of hypothyroidism. Menses started on May 13 and on June 6, 1948. Vaginal smears showed ovulation between June 22 and 24 and there was a distinct rise in temperature at this time. Smears showed maximal cornification 60%. The luteal phase was poor with scant secretion and little mucus. Calorimeter temperature was 24.7 on June 3, 1948. Cold, had waves of chilliness and goose flesh but did not shiver. Temperature was 26.5 on May 27. Feet cold. Temperature was 28.0 on May 25. Temperature was 30.9 on June 1. Quiet, axillae and forehead moist.

Figures 1 and 2 give a general picture of the changes in metabolism in calorimeter temperatures from 22°C. to 35°C. Inasmuch as the calorimeter walls are in thermal equilibrium with the air, these are equivalent to the "operative" temperatures of Winslow, Herrington and Gagge ('37). The points on the curves give the average of the first and second hours except in those cases where only the first hour was

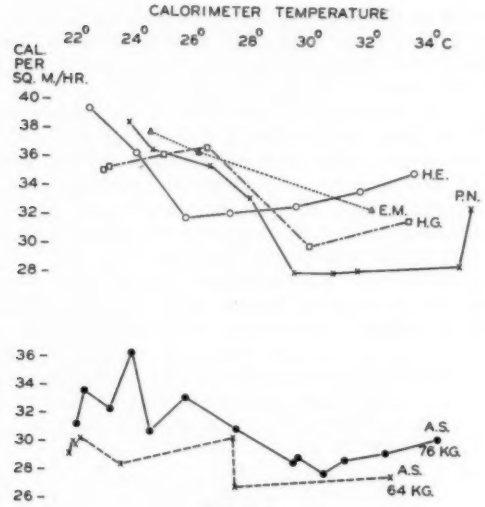

Figure 1

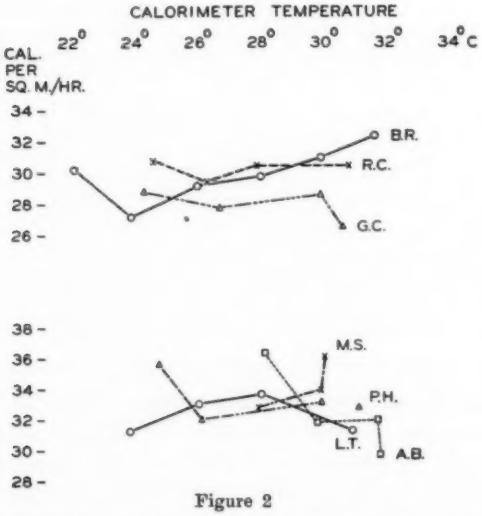

Figure 2

Figs. 1 and 2 Basal metabolism at calorimeter temperatures from 22–35°C. averages of first and second hours except in a few instances when second hours were not available.

basal. Individual differences are brought out clearly. The first graph shows 4 women whose metabolism was high in the cold zone, the second shows the metabolism for A. S. in 1939 at 76 kg, and the lower and flatter curve in 1940, when she was on a reducing diet.

Figures 3–8 give the details of the 5 women who were studied in 6 or more experiments. Figure 9 charts the respiratory quotients in all of the experimental hours. All are in the range to be expected from the previous diet and length of fasting.

Figure 10 is a smoothed curve giving the heat production calculated from oxygen consumptions in the first and second hours. Seventy-six experiments on 13 women were available. The need for smoothing is seen from the irregular distribution of measurements over the temperature range. For example, there were only two experiments between 25.0 and 25.9, but 11 at 24.0 and 24.9, and 10 at 26.0 and 26.9. The average level for 25° was therefore determined by adding the totals for 24°, 25°, and 26° and dividing by 23. The whole curve was smoothed by using three degrees except at the extremities where only two degrees could be employed. Trial curves were made using the averages of all experiments but it became evident that this was unfair. For example, the 6 experiments on A. S. far outweighed the one for H. E. plus one for B. R. The curves in figure 10 were obtained by using the averages of the individuals, e.g., three at the 22° level.

Heat production of women at different environmental temperatures

From an inspection of figure 10 it is apparent that the metabolism is distinctly higher in the second hour than in the first hour. There is also evidence that in the first, or basal, hour the metabolism is lowest in the region of 30–32 with a tendency to be higher in the warm zone and distinctly higher in the cold zone. The two extremities of the curves do not show these trends. Only three individuals, H. E., A.

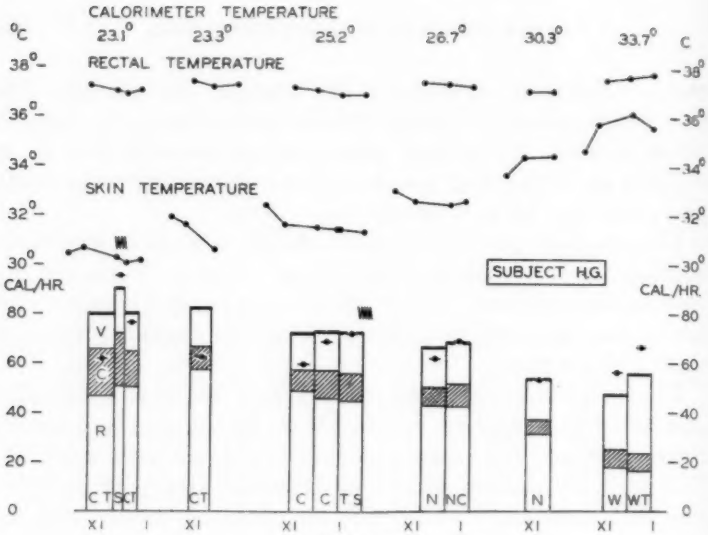

Figure 3

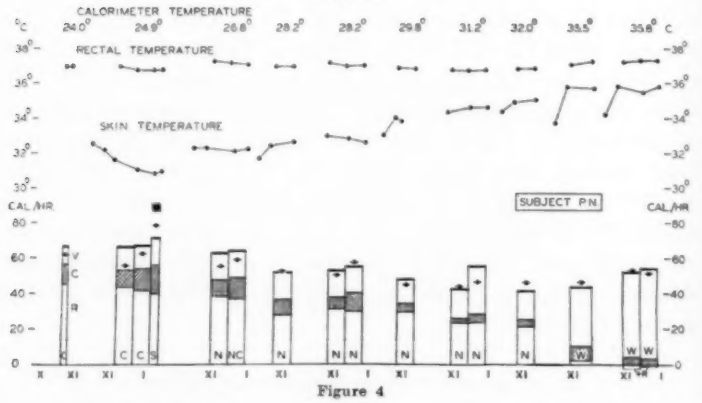

Figure 4

Figs. 3–8 The top line gives the average temperature of the calorimeter at the time of the experiment. The skin temperatures are the average of readings of each part of the body weighted according to the effective radiating area of the part. The black dots with short horizontal bars represent the heat production as determined by the method of indirect calorimetry. The columns show the total heat loss as measured by direct calorimetry. The top division of each column gives the loss by vaporization (V); the hatched portion by convection (C); and the lowest division, radiation (R). At the base of each column letters indicate the sensations of the subjects: S, shivering (also marked by zigzag lines above the column); C, cold; C T, cold with tension in a few muscles; N, neutral, or comfortable; W, warm. The Roman numerals at the base show the time of day.

S. and B. R., were studied between 22 and 23.9. Their basals in the comfort zone averaged 2% below the graph as a whole. In the range from 34 to 35.9 there were only two, A. S. and P. N., with an average basal 4% below the group. The preponderance of women with low metabolism at the two extremities accounts for part, if not all, of the changes in the direction of the curve.

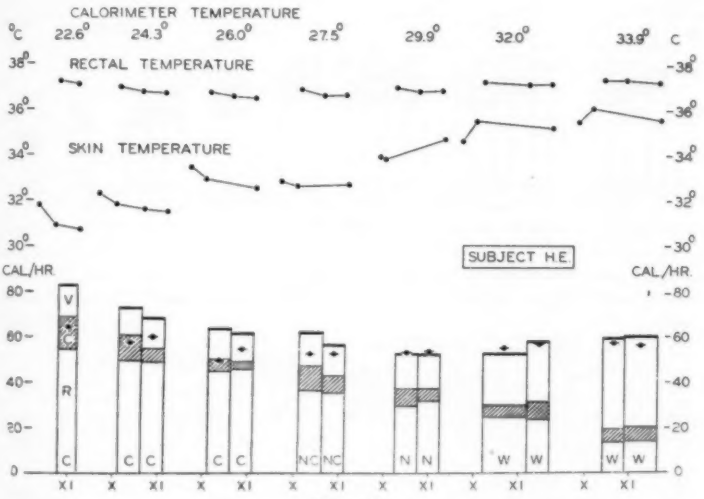

Figure 5

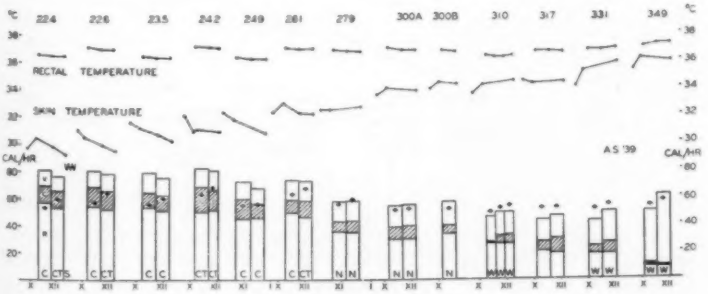

Figure 6

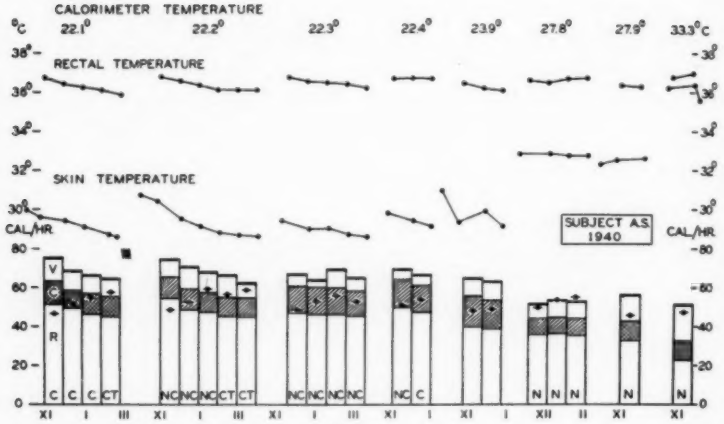

Figure 7

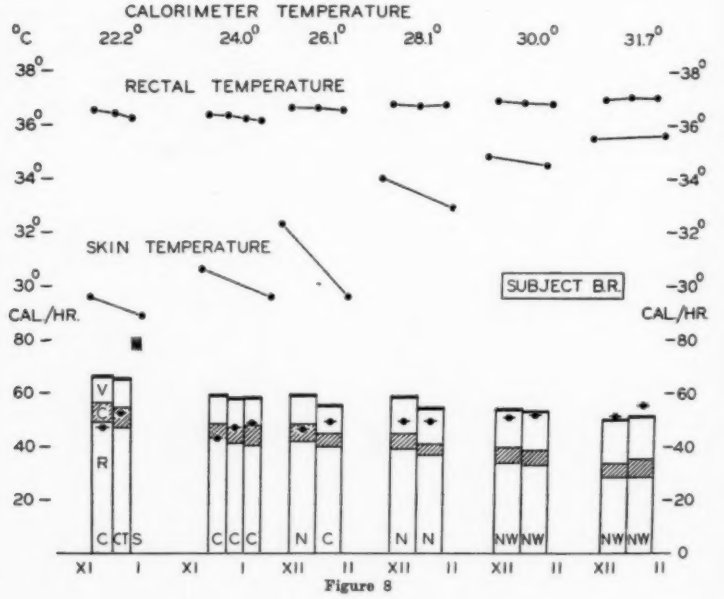

Figure 8

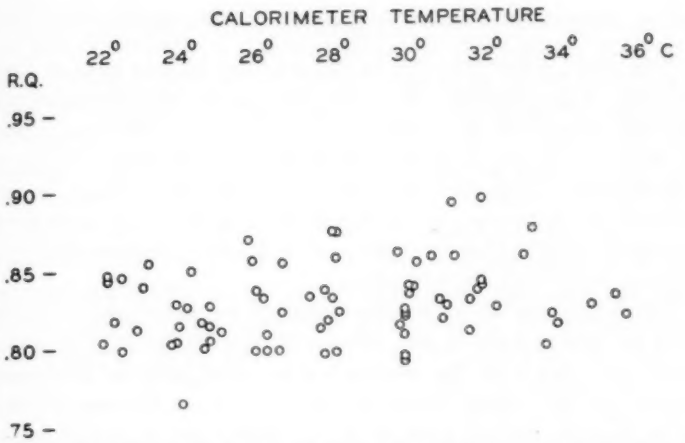

Fig. 9 The average respiratory quotients, in most cases the average of two experimental hours. Three experiments have been omitted on account of technical difficulty in measuring oxygen. One was omitted because the woman had eaten coffee cake at 9 the previous evening. The average quotient indicates that the calories derived from carbohydrate and fat were about equal. The protein calories were low.

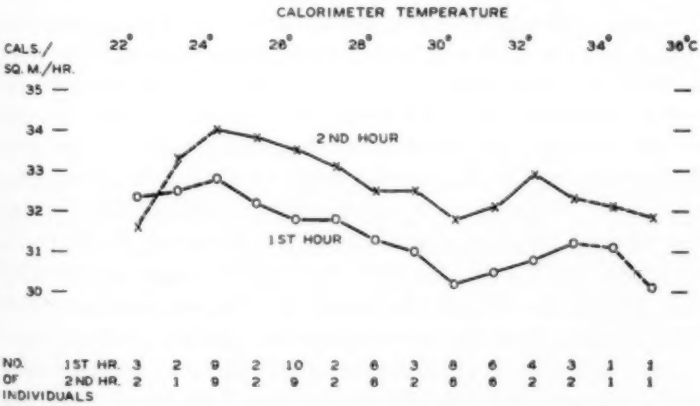
CALORIMETER TEMPERATURE
CALS./ SQ. M./HR.
22° 24° 26° 28° 30° 32° 34° 36°C
35
34
33
32
31
30
2ND HOUR
1ST HOUR
NO. OF INDIVIDUALS
1ST HR. 3 2 9 2 10 2 6 3 8 6 4 3 1 1
2ND HR. 2 1 9 2 9 2 6 2 8 6 2 2 1 1

Fig. 10 Smoothed curve giving the average heat production in terms of calories per square meter per hour. The circles represent the first hours only, the crosses the second hours. The average metabolism of each individual is averaged with those of other individuals at the same and at two adjacent temperatures.

Rise of heat production in the second hour

The causes for the rise of metabolism in the second hour are not entirely clear. In some, but not in all, of the second periods, there was the factor of measuring the surface. There is also the factor of increased length of exposure to an uncomfortable environment with boredom and slight, undetected, restlessness. In the cold and warm zones there is the increased effect of adjustment to the environment, the factor we are trying to measure.

In the series of 13 women studied in 1938 and 1948 the rise of metabolism in the second hour averaged 5.9% in the cold zone, 3.4% in the comfort zone, and 3.2% in the warm zone. In the range from 22–35 there were 43 experiments with second hours, and in 33 of these pairs, there was a rise in the second hour. Only 10 showed a fall. Statistically the chances of this occurring by accident are about one in 1,000.

This tendency towards a rise in metabolism in the second hour is an old phenomenon in the Sage respiration calorimeter. It is shown in the records of experiments made between 1913 and 1932 before the introduction of surface temperature measurements. The subjects, lightly dressed, were very comfortable in environments of 23 to 24. It was possible to find 31 satisfactory experiments on 19 normal men and two normal women. In 19 of these basal experiments the heat production rose in the second hour, in 12 it fell. Averaging all of these with regard to sign the figures indicate an average rise of 1.4%. In the case of the normal control, E. F. D. B., who was studied most often, the average rise before 1932 was only 0.6%. After 1936 when he was exposed naked to various temperatures, measuring the surface temperatures in some but not all of the experiments, the rise in the second hour averaged 3.0% in the cold zone, 3.1% in the comfort zone, and 4.3% in the warm zone.

Many others have noted a changing metabolism during the experimental periods. Berkson and Boothby ('38) have made

a careful analysis of their records with short periods using the Tissot technique. "For the first two or three observations (which will mean on the average until about 9:30 A.M.) there is a decrease in metabolism amounting on the average to about 0.4 cal. per square meter. Then there is a rapid and continuous rise to above the initial value so that by the 7th observation (about 12 noon) the mean increment of metabolism is positive and about 0.4 cal. per square meter. We may speculate that the initial lowering is correlated with an increasing adjustment to basal conditions and that as time passes, restlessness begins to be effective in raising the metabolism." They found that for the 8th and 9th observations the rise was about 1.0 to 1.4 cal. per square meter.

The differences of about 3% are not great and most investigators feel happy when two consecutive determinations of the basal or "standard" metabolism come within 5%. It is only the strong preponderance of increases in the second hour that inclines us to take the first calorimeter hour as the truer index of the basal. The average 5.9% rise of the women in the cold zone as opposed to a 3 or 4% rise in the other zones indicates, but does not prove, that the prolonging of the exposure to cold is a factor which increases metabolism. A study of the continuing adjustment of loss by radiation supports this view.

Body temperatures

Figure 11 gives the curves for average rectal and surface temperatures smoothed by averaging the results at each degree with those of the adjacent degrees above and below. The line for the average rectal temperature is surprisingly level at 36.8° to 36.9°C. from environmental temperature of 25 to 32. It is slightly lower below 25°C., and higher above 32°C. A study of the individual graphs shows a tendency for falling rectal temperatures during any experiment in the cold zone, a level or slightly falling in the comfort zone, and a rising or level temperature in the warm zone. The level or falling rectal temperature in the comfort zone con-

firms the statement of DuBois ('41) that the usual morning rise of body temperature is absent under strict basal conditions.

The rectal temperature measurements obtained by the Sage calorimeter technique are probably more uniform than those of any other series in the literature for several reasons. The

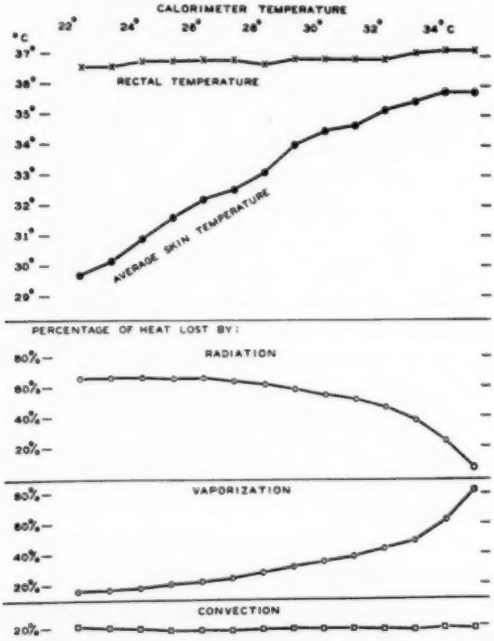

Fig 11 Smoothed curves giving averages in all experiments during the first experimental hour.

thermal element of 100 ohms resistance is encased in Woods metal in a silver tube 6 cm long and 8 mm in diameter. This is attached to a moderately flexible rubber tube and is inserted so that the tip is in the sigmoid flexure 12 cm from the anus. This is at the depth which, according to Benedict and Slack ('11), and Mead and Bonmarito ('49), gives the maximal readings. X-rays of the Sage thermometer taken with two

normal men have shown that it lies almost exactly in the center of the lower pelvis where it would be least influenced by blood flowing from the legs. There must be some influence from the cooled blood returning from the legs especially in the cold zone; it is impossible to measure exactly how much without using thermometers in the abdomen and thorax.

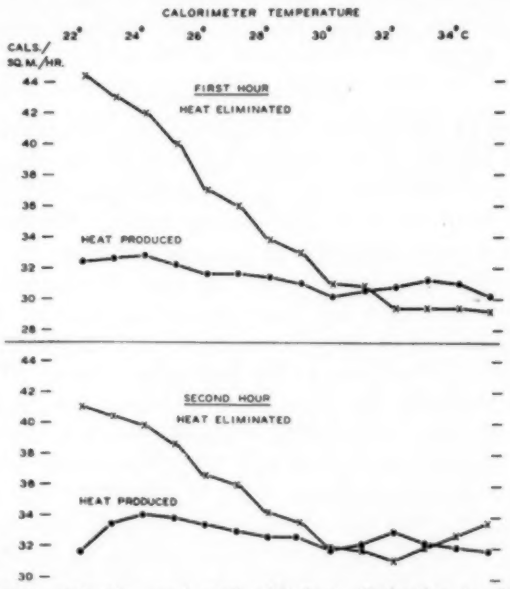

Fig. 12 Heat production contrasted with heat elimination in the first and second experimental hours.

Surface temperatures. Figure 11 shows the smoothed curves of average surface temperature with its steady rise in environments from 22°C. to 29°C., then a slower rise with a leveling at 34°C.–35°C. The graphs of individual experiments show falling skin temperatures in the cold zone, rising in the warm zone, with variable changes in the comfort zone.

These surface temperatures were measured by the Hardy radiometer in 20 parts of the body and were then weighted for the effective radiating areas of each region. A recent

careful comparison of surface thermometers (Stoll and Hardy, '49, '50) has shown that the radiometer is the most reliable instrument. There is of course a wide variation in the different parts of the body, as was demonstrated in the experiments on men (Hardy and DuBois, '38). The estimations of conductance through the peripheral tissues and the average body temperature have been discussed in the earlier reports on women (Hardy and DuBois, '40; Hardy, Milhorat and DuBois, '41).

Heat elimination

Figures 11 and 12 give the relationship of heat production, heat loss and the percentage of calories lost in radiation, convection and vaporization. Here again smoothed curves are used for both the first and second hours. Naturally in the cold zone a great deal of heat is being lost from the periphery of the body. In the first hour the heat elimination at 22°C. is 37% greater than the heat production, and at 26°C. it is 17% greater. It is only between 30 and 32.9 that they are approximately the same in the first hour. Above 32 the elimination lags behind the production and does not catch up until the second hour. It must be remembered that the first calorimeter hour starts one or two hours after the women have undressed and commenced their exposure to the particular environment. The loss of heat in the cold and the adjustments in the cold and warm zones change as the hours progress. This is shown by the fact that in the cold zone the heat eliminated in the second calorimeter hour is distinctly less than in the first hour in spite of an increased heat production. In the warm zone the heat elimination increased during the second hour, showing a tendency to balance the accumulation of heat in the first hour.

A study of the details of heat loss explains the mechanism of these changes in the second hour. At temperatures of 23 and above, the percentage lost by radiation decreases slightly with falling skin temperatures and the percentage lost by convection increases slightly. In the warm zone the percentage lost by vaporization increases during the second hour.

The smoothed curves in figure 11 demonstrate a surprising uniformity of the average loss by convection although the differences in individual hours are relatively large. The fact that the average rise of convection in the second hour is small indicates that the subjects had little increase in their movements during the second hour. A paper by Hardy, Milhorat and DuBois ('38) demonstrated the marked increase in convection with moderate exercise or chills.

When the environmental temperature is nearly the same as the skin temperature there can be little or no loss by radiation and convection. Vaporization takes care of all of the loss and in doing so keeps the skin temperature far enough below the core temperature to provide a gradient between the core and the periphery.

DISCUSSION OF RESULTS

Basal metabolism

The average basal metabolic rate of the 13 women with an average age of 25.6 years was 31.2 cal. per square meter per hour in the comfort zone, and it so happened that it was the same for the first and second series. The different individuals showed considerable variations in their averages. The lowest was G. C. at 27.6, the highest 34.5 in the first and only observation on H. H. The estimate of the average BMR for women of this age made by Aub and DuBois in 1917 was 37.0. In 1936 Boothby, Berkson and Dunn gave as standards 36.18 for the ages 20–24 and 35.70 for the ages 25–44. Since that time there have been many reports on normal women with figures that are much lower. The excellent new standards of Robertson and Reid ('52) give an average of 33.9. All of the women of this series may be considered to be within the normal range.

The effect of environmental temperature on basal metabolism

There are available in this series 76 experiments on 13 women and it is possible to make a more accurate analysis

of the effect of the environment on metabolism. The important points are whether or not an effect is demonstrable, and if so, the cause or causes of this effect.

The shapes of the average curves and the statistical analysis show a significant small rise of metabolism in the cold zone and a trend towards a rise in the warm zone. The causes are still uncertain.

The literature on the "chemical regulation" of Rubner and the effect of environment on men has been reviewed in our previous publications (Hardy and DuBois, '40; Hardy, Milhorat and DuBois, '41). The generally accepted view that there is no significant change in the basal metabolism of naked men in short exposures at temperatures from 22 to 35 is supported by much fragmentary evidence. It is, however, possible that if there were a study on men as comprehensive as this one on women, significant trends might be demonstrated. Unfortunately there are respiration calorimeter studies on only two normal men in this range of temperature (Hardy and DuBois, '38).

In addition to the publications quoted in our previous papers there are a few old studies and many recent ones that deserve attention. In general most writers support the view of little or no change in the metabolism of men in the range from 22 to 35. Almost everyone emphasizes the great variability in subjects. Some say that metabolism in the cold can rise without increase in muscle movements, others deny this. The recent German literature tends to include under Rubner's term "chemical regulation" factors such as chills and sweating whereas the Americans exclude these and limit the term to effects produced by hormones, emotions or effects similar to the specific dynamic action of foods.

Voit in 1878 was a pioneer in the study of cold. Loewy in 1890 published a fine study of men at different temperatures under basal conditions. There were 55 experiments. In 9 of these the oxygen consumption fell in the cold, in 20 it changed less than 5%. In 26 the oxygen rose in the cold. In half of these there was shivering or tension, in the other half no

shivering or tension was observable but Loewy believed that the rise in the cold should be ascribed to small twitches that cannot be detected by visible muscle contractions. Loewy recognized the importance of contraction of the peripheral blood vessels as a means of diminishing the loss of heat from the body. McConnell and Yagloglou in 1925 noted that the minimal metabolism of men was in the range between 75°F. and 83°F. "effective temperature." Horvath, Golden and Wagar ('46) placed a few men in air-conditioned rooms at different temperatures for two to 6 days. There was little evidence of any marked change in energy metabolism. Park and Palmes ('48) in a study of fever using a new and rapid type of calorimeter did not obtain data in favor of a "chemical regulation." All augmentations of heat production were caused by chills or were secondary to elevation of body temperature. In three out of their 4 graphs showing chills the metabolism remained level up to the onset of shivering. In the 4th case the body temperature and metabolism had risen slowly before the onset of chill.

In 1940 Dr. Eleanor Mason (E. M. of our series) studied in 21 women the effect of a change in residence from a temperate to a tropical climate. She found two types of women. Thirteen in type 1 showed a drop of 6 to 11% in the tropics. Eight in type 2 had little or no change. In 1944 Dr. Mason determined her own basal metabolism daily on a voyage from Honolulu to the tropics. Leaving Hawaii her BMR was 30.1–31.2 cal. per square meter per hour. Ten days later in the tropics it had fallen to 28.1. In 1938 Hick, Keeton and Glickman studied one man and 4 women at effective temperatures between 60°F. and 95°F. Judging from their graphs two of the women showed an increase in metabolism in the cold amounting to 6–10%, and all had an increase when very warm.

Important work on temperature regulation has appeared in the German literature. Thauer and Wezler ('43), using a climate chamber with rapid interferometer readings of oxygen and carbon dioxide, made 48 observations on three nor-

mal young men and one young woman for three-hour periods under basal conditions. The metabolism curve was level in their "comfort zone" 22°C.–38°C. Above 38, the heat production rose sharply. Below 22, the rise was variable depending on the amount of shivering. One man showed a curious dip of metabolism between 34°C. and 36°C. where the level was about 10% lower than at 28–30. Gollwitzer-Meyer ('37) had noted a similar drop in a warm bath that caused complete relaxation. The fall in metabolism in the one subject of Thauer and Wezler is about the same as the 10% drop during sleep noted by Mason and Benedict ('34). The woman subject in Thauer's experiments for the first hour at 15°C. had a distinct rise in metabolism without trace of shivering or chills. Wezler and Thauer ('43) compared the vaporization loss of one man and one woman at 50°C. and 50% humidity. The woman lost only 1.01 kg, the man 1.61 kg, and his curve of water loss started to rise sooner and was much steeper. König ('44), using the same apparatus, studied 8 more men naked or in swimming trunks. He found great variations. One of the men (Büttner) had no rise in metabolism in the room at 13°C. König believes that without muscle contraction there is no rise in metabolism. Wezler and Neuroth ('49) again using the same apparatus studied 4 men and two women but it is hard to interpret their results as the time of chills is not indicated. They tried to calculate the fall in metabolism that one would expect in the peripheral tissues as the peripheral temperature fell (Van't Hoff effect). They call attention to the fact that if there is a reduction in the peripheral zone there must be a chemical regulation increase in the core of the body.

Tensing and the approach to chills

Figure 13 reproduces three chill experiments on men from the report of Hardy, Milhorat and DuBois ('38). In all three experiments the metabolism remained at or near the man's average basal level up to the onset of the chill. Figures 3

to 8 show that the women had a rising metabolism in the hours preceding the chills.

When the body cools to a certain point there is an involuntary tensing of the muscles in the trunk or in one extremity after another. Often this ceases in a few minutes but if the body is cool enough and if the man cannot control him-

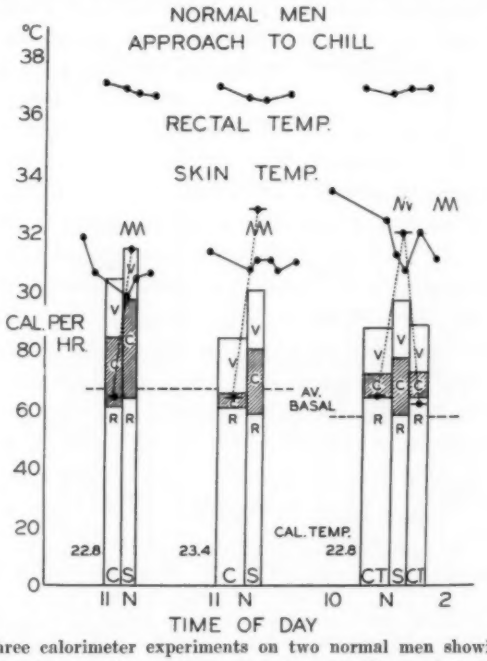

Fig. 13 Three calorimeter experiments on two normal men showing that the metabolism was close to the man's average basal in the period just before a chill.

self the mild tensing changes abruptly into frank shivering. The important points at question are: (1) does the subject always appreciate the onset of tensing; (2) can it always be detected by a careful observer; (3) can it be detected by instruments; and (4), and the most important, does it cause a significant rise in metabolism? Opinions are divided on all of these points.

We believe that our subjects, who were all intelligent and carefully trained, gave us accurate reports on the presence or absence of tensing. Almost everyone is agreed that it is often impossible for an observer to detect tensing since it may occur without visible movement of the extremity. The

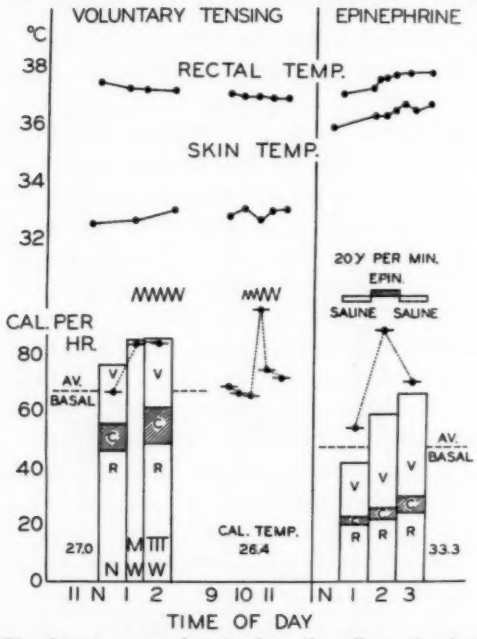

Fig. 14 The first two experiments show the effect of voluntary tensing of the muscles on the heat production. In the first experiment there was vigorous tensing in the second and third periods. In the second experiment the first two short periods were basal, in the third the tensing was mild, in the 4th it was vigorous. The third experiment illustrates the action of epinephrine.

most sensitive type of instrumental detection needle electrodes as used by Burton and Bronk ('37) in their preliminary report on cats showed twitches of single muscle units. Myographic tracings have been made by several investigators. Hemingway and Hathaway ('41) made a careful study of three well-trained dogs with electrodes over the muscles and

with the hind quarters on a sensitive balance. Their basal metabolism rates during the comfortable control periods averaged 34 cal. per square meter per hour. The temperature was then dropped 5°C.–6°C., the metabolism rose 5 to 10% (average 7) before shivering and 24–42% during shivering. The electrodes picked up the onset of shivering very definitely. These authors conclude that the chemical regulation without any sign of shivering is only 7% on the average and is so small that it is without practical significance in protecting against exposure to cold.

An experiment at Woods Hole was performed on one of us through the courtesy of Dr. John F. Perkins, Jr. Sensitive electrodes on 6 parts of the body showed almost no change during mild tensing before a chill but vigorous response at the onset of real or simulated chill.

One attempt to evaluate the effect of maximal tensing by Swift ('32) showed an increase of 36% in metabolism. Another attempt made by one of us has been described by Hardy, Milhorat and DuBois ('38) and the results are shown in figure 14. After one basal period the man started tensing his muscles as hard as he could, first in one extremity and then in another, and kept this up for 50 minutes. At the end he was very tired but he had increased his heat production only 25% and his elimination 11%. In 1950 more tests were made using a Benedict-Sanborn apparatus with 6-minute periods. The results are shown in the middle of figure 14. The first two periods were basal. In the third period he imitated every 15 seconds the slight muscular tensings that precede a chill using first one arm, then the other and then each leg. In spite of this the level of the metabolism was practically unchanged. In the 4th period he exerted severe tensing for 5 seconds, relaxing 5 seconds, and kept this up for 10 minutes. The metabolism rose 42% and then fell rapidly after he stopped. In figure 14 is also shown the sharp rise in metabolism of a normal man in the calorimeter when a small amount of epinephrine was added to

his intravenous saline infusion. The stimulating effect of epinephrine was studied extensively by Sandiford ('20).

The effects of tension of muscles and rigidity have not received the attention they deserve. Roaf in 1912 and 1913 abolished the decerebrate rigidity of cats by means of curare and by cutting the nerves to all 4 limbs. There was no significant reduction in the carbon dioxide output and only a slight fall in the oxygen consumption. Dusser de Barenne and Burger ('24) studied cats soon after decerebration and then "abolished rigidity" by cutting the sciatic and femoral nerves and the brachial plexus. The drop in metabolism was small.

Grafe ('20–'23) discussed muscle tone and total metabolism, reviewed the literature and cited his own experiments on cataleptic spasm in hypnosis and spastic paralysis. He concluded that a tension of muscles does not lead to an increase in oxidation, as long as there is no significant work performed.

Statistical analysis

The statistical analysis of the results has been difficult and many methods have been explored.[3] It happens that the simplest method has given the most satisfactory results. It is apparent from figures 1 and 2 that there is a distinct tendency for a metabolism higher in the cold than in the comfort zone. The probability that this is not due to chance was determined with the aid of table 2. There were 10 cases where the metabolism was measured both in the comfort and in the cold zones (H. G., P. N., A. S., 1939; A. S., 1940; H. E., B. R., G. C., P. H., L. T., and R. C.). It is necessary to list A. S. as two different women since her weight was 76 kg in 1939 and 64 kg in 1940.

In each case the woman's basal in the comfort zone was used as a standard for comparison. In 4 cases it was determined from the average of the first and second hours of

[3] We are greatly indebted to Dr. Donald R. Charles of Rochester University, Rochester, New York, for his help in this analysis

one calorimeter experiment, in 6 cases from the average of the first and second hours of two or three experiments. In table 2 are tabulated the number of times the tests in the cold zone were higher (+) or lower (—) than the standard for that woman in the comfort zone. It will be noted that in the "cold zone" (22°C.–26.9°C.) out of a total of 32 experiments the metabolism was higher 23 times and lower 9

TABLE 2

Cold zone deviations from each woman's average in comfort zone

CAL./SQ. M PER HOUR	"COLD ZONE" 22°C.–26.9°C.		ZONE 22–24.9	
	+	—	+	—
0–0.9	3	4	2	2
1–1.9	6	3	6	
2–2.9	2	1	2	1
3–3.9	3	1	2	1
4–4.9	1			
5–5.9	3		3	
6–6.9	4		2	
7–7.9	1		1	
No.	23	9	18	4

TABLE 3

Ten women's basals in comfort zone

CAL./SQ. M PER HOUR	DEVIATIONS FROM AV. 30.7	
	+	—
0–0.9	1	4
1–1.9	1	
2–2.9	1	1
3–3.9	1	1

times. The probability (P) that this would occur by chance is 0.01. Five of the 9 minus figures came between 26°C.–26.9°C. which is the edge of the comfort zone. If we consider the 22 tests between 22 and 24.9 there are 18 above the comfort zone standard, and only 4 below; $P = 0.002$. The average rise in metabolism in the zone below 22°C.–24.9°C. was 7.6% (s.d. 7.5%; standard error of the arithmetric mean 1.6%). Table 3 shows the rather small deviations of the

comfort zone standards of these 10 women from their average of 30.7 cal. per square meter per hour.

At present the evidence is that the slight muscle contractions reported as tensing by our subjects have so small an effect on metabolism that they cannot account for all of the rise found in some of the women in the cold zone. A small increase in the release of epinephrine or some similar substance could account for the rise but there is little evidence for or against epinephrine effect. There is no evidence that the increase is due to anything resembling the specific dynamic action of foods. The nitrogen excretion of the women remained at a low level; the respiratory quotients were very uniform. We are left, therefore, with the presumption that in some but not all women there is a true chemical regulation in the old Rubner sense but we must agree with Hemingway and Hathaway ('41) in their conclusion that it is so small that it has little practical significance in protecting against severe exposure to cold but might be helpful in mild exposure.

Van't Hoff effect on basal metabolism

Recently several investigators have discussed the question of making allowances for differences in body temperature when calculating the basal metabolic rate. A good many years ago it was shown (DuBois, '21) that in fever the metabolism is raised about 13% for each degree Centigrade rise in rectal temperature. Benedict, Benedict and DuBois ('25) pointed out the probable increase of metabolism in peripheral tissues warmed in hot air baths. What we need to know in our experiments on women is the temperature of the peripheral tissues in cold environments, the core temperature and the average body temperature.

Figure 11 shows the rectal and average skin temperatures, and from them the average body temperature can be approximated by using the formulas of Hardy, Milhorat and DuBois ('41). At all temperatures the rectal temperature is assigned the greatest weight and the skin temperature, rep-

resenting the peripheral tissues, weights depending on the depth of cold penetration. The skin temperature is weighted as follows: Environments 22°C.–25°C. 40%, 25°C.–28°C. 30%, 28°C.–33°C. 20% and 33°C.–36°C. 10%. This allows one to make a curve of "average body temperature" from 33.8°C. at 22, to 37.1°C. at 35. In the cold region 22–25 the average body temperature is 34.1°C., in the comfort zone 36.3°C. and in the warm zone 37.0°C.

Since in the cold region the average is 2.2°C. lower than in the comfort zone we might expect 2.2×13 or 28.6% lowering of the standard basal metabolism, and in the warm zone 0.7×13 or 9.1% rise. The fact that the metabolism in the men and some of the women does not change significantly with environment shows that there must be a chemical regulation which increases metabolism in the core of the body to make up for the lowering in the periphery.

The calculations will never be exact because we do not know the percentage of metabolism that is derived from the peripheral tissues. There is reason to believe that the viscera in the core of the body have a much higher rate per kilogram than the skin, subcutaneous tissue and resting muscles of the periphery. If we assume only half the values of the previous calculation this would indicate a chemical regulation by the internal organs which amounts to an increase of 14% of the total metabolism in the cold before any signs of shivering.

Comparison of the mechanics of temperature regulation in men and women

A summary of the differences between the sexes in the mechanics of heat loss is presented in figures 15 and 16. This is only a small segment of the many differences between men and women for which we are all grateful.

Between the calorimeter environments of 24°C. and 33°C. there are enough experiments on women to give satisfactory statistics. The two extremes of the lines are based on fewer subjects. Unfortunately there are only two men available for

comparison under exactly similar conditions. Both had been found close to the average normal in many kinds of metabolic experiments. J. D. H. at the age of 33 in the calorimeter series, had an average basal rate 12% below the Boothby, Berkson and Dunn ('36) "standards." E. F. D. B. at the average age of 55 was 7% below the standards. These are both

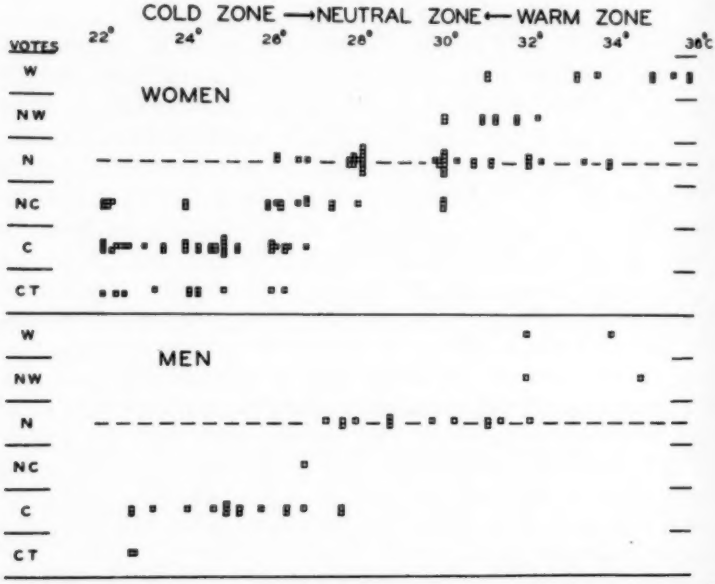

Fig. 15 Comfort votes of 13 women and two men in the environmental temperatures from 22° to 36°C. The votes for both the first and second hours are recorded except in those cases where only one basal hour was available. W, too warm; N. W., neutral to warm; N, neutral (comfortable); N. C., neutral to cold; C, too cold; C. T., cold with tensing of a few muscles.

very close to the average now found with modern basal technique in physiological studies.

The comfort votes of the men and women are shown in figure 15 and, in general, the differences appear small. The women seem to have a somewhat wider comfort zone. Between 28 and 31 all of the women and men were comfortable

or nearly so. In this zone the average skin temperature of the women was 33.9°C.

Figure 16 compares the smoothed curves for the 13 women and the two men. The figures are taken from the first experimental hours only except in the case of J. D. H. whose basal

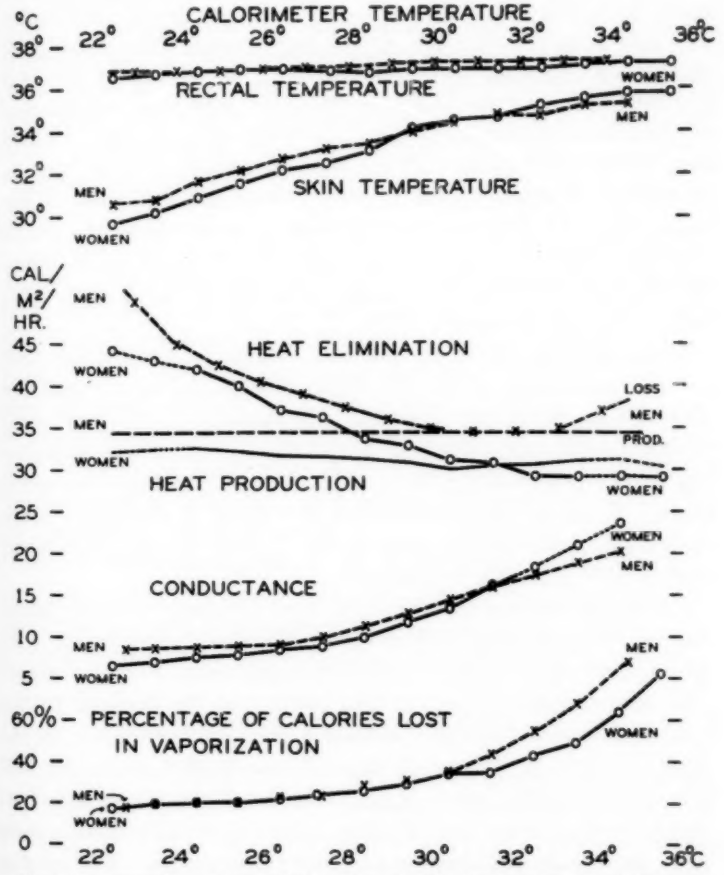

Fig. 16 Comparison of smoothed curves of data for 13 women and two men. Only the first experimental hours are used. The dashed line shows the heat production of the men which was level throughout the temperature range.

metabolism was calculated from the average of the first two hours in the few experiments when he slept part of the first hour.

The rectal temperatures at the top of the chart are practically identical, both only 0.3°C. lower in the cold than in the comfort zone. The skin temperatures of the women were about a degree lower than those of the men in the cold zone and half a degree higher in the warm zone. The heat production of the men was level throughout the range at 34.5 per square meter per hour. In the cold zone the average for the women was about 7% lower than the men, in the comfort zone about 12% lower. There was, however, a wide scatter. In the 42 separate hours in which women were studied between 22.9 and 25.2, 6 showed metabolic rates above the range 32.8–36.1 (cal. per square meter per hour) found in the men. Eleven were within this range and 25 of the hours were below the men's range.

Heat elimination of women was distinctly lower than that of men especially at the two extremes of the curve and the loss of heat from the bodies of the subjects in the cold zone (negative storage, heat loss minus heat production) was less for the women than for the men, especially during the second hours. Even so, in the women it averaged 10 cal. per square meter per hour in the zone from 22–24.9 in the first hour, and 18 for the first two hours.

Conductance (conductivity) of heat through the peripheral tissues was calculated by dividing the heat elimination (cal. per square meter per hour) by the difference between rectal and skin temperatures. It was lower for the women in the cold and comfort zones showing a greater resistance to the loss of heat. In the warm zone it was higher and they lost heat more easily. One would assume that the lower conductance of women was due to a thicker layer of subcutaneous fat. This may not be the correct explanation. The young woman A. S. at temperatures between 22 and 23 in 1939, when she weighed 76 kg, had an average conductance of 7.45. In

1940 after she had lost 12 kg it was 5.65. The reason for this unexpected change is not clear.

The percentage of calories lost in vaporization was identical until the environmental temperature was over 31. Then the women did not need to sweat as much as the men.

Other differences between men and women were shown in the approach to chills. The women were able to stand exposures to the cold much longer than the men before they started to shiver. There were 8 experiments with chills in women, 6 with men. Just before shivering the average rectal temperatures were identical, 36.7°C., but the women had tolerated an average skin temperature 0.3° lower than the men.

A summary of all these rather small differences adds up to the physiological advantage of the women in both the cold and warm zones. In the cold zone most of the women can increase their heat production without muscular movement. Their skin temperatures are lower and they lose less heat through the skin and subcutaneous tissues. They have a wider comfort zone. In warm environments their metabolism is so much lower than that of the men that they do not need to lose as much heat per unit of surface and, therefore, do not need to sweat as much.

In general this confirms the conclusions reached in our previous report (Hardy, Milhorat and DuBois, '41) but the differences are less marked. This is due to the inclusion of 7 more women, and slightly different methods of calculation.

SUMMARY AND CONCLUSIONS

Seventy-six experiments in the Sage respiration calorimeter were made on 13 normal young women at environmental temperatures between 22°C. and 35°C. The measurements in hourly periods included heat production, respiration quotient, rectal temperature, average surface temperature, total heat loss, conductance and the percentages of heat lost by

radiation, convection and vaporization. Careful note was made of muscle tensing, shivering and sensations of cold, warmth and comfort.

The average basal metabolism of the 13 women was 31.2 cal. per square meter per hour. This is so much lower than the heat production of men per unit of surface that women have an advantage over men in the warm zones and do not need to sweat as much. In the cold zone some, but not all, women lose heat less rapidly than men.

In the cold zone the women lost about 67% of their heat by radiation and this percentage dropped in linear fashion to zero when the calorimeter temperature was the same as skin temperature. Vaporization accounted for only 16–18% of the total loss at the coldest temperatures and reached the conventional figures of 24–25% only at environments of 27°C.–28°C. It is obvious that fixed percentage figures for radiation, convection and vaporization apply to only one strictly specified set of conditions.

The comfort or neutral zone for women, naked, and in strict basal conditions, extended from about 26°C. to 30.9°C. In this zone the average skin temperature was 33.9°C. and in the upper part of the zone heat production closely approximated heat elimination. Of the 10 women studied at temperatures of 28 and below, 6 showed an increase in metabolism in the cold greater than the amount that could be ascribed to muscle tension or restlessness. This is evidence that some but not all women are subject to Rubner's "chemical regulation" of metabolism but the amount involved is small.

In the cold zone the peripheral tissues lost so much heat that the average temperature of the body dropped 2.8°C. before the onset of shivering. The metabolism of the peripheral tissues falls with lower temperatures. Since the total metabolism remains constant or rises there must be an increase in the metabolism of the core of the body which compensates for the decrease in the periphery. In this sense there is a chemical regulation in men and women.

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OSBORNE AND MENDEL AWARD

Nominations are invited for the Osborne and Mendel Award of $1000.00 established by the Nutrition Foundation, Inc., for the recognition of outstanding accomplishments in the general field of exploratory research in the science of nutrition. It shall be given to the investigator who, in the opinion of a Jury of Award, has made the most significant published contribution in the year preceding the annual meeting of the Institute, or who has published a series of contemporary papers of outstanding significance.

The Award will be presented at the annual meeting of the American Institute of Nutrition.

The recipient will be chosen by a Jury of Award of the American Institute of Nutrition. As a general policy, the Award will be made to one person. If, in the judgment of the Jury of Award, an injustice would otherwise be done, it may be divided among two or more persons. Normally preference will be given to research workers in the United States and Canada, but investigators in other countries, especially those sojourning in the United States or Canada for a period of time, are not excluded from consideration. Membership in the Institute of Nutrition is not a requirement for eligibility and there is no limitation as to age.

Nominations may be made by anyone. Nominations for the 1953 Award, accompanied by data relative to the accomplishments of the nominee, must be sent to the Chairman of the Nominating Committee before January 1, 1953.

Chairman, Nominating Committee:

DR. PHILIP HANDLER
Department of Biochemistry and Nutrition
Duke University School of Medicine
Durham, North Carolina

BORDEN AWARD IN NUTRITION

Nominations are solicited for the 1953 Award and a gold medal made available by the Borden Company Foundation, Inc. The American Institute of Nutrition will make this award in recognition of distinctive research by investigators in the United States and Canada which has emphasized the nutritive significance of the components of milk or of dairy products. The award will be made primarily for the publication of specific papers, but the Jury of Award may recommend that it will be given for important contributions over an extended period of time. The award may be divided between two or more investigators. Employees of the Borden Company are not eligible for this honor.

The formal presentation will be made at the annual meeting of the Institute in the spring of 1953. To be considered for the award, nominations must be in the hands of the Chairman of the Nominating Committee by January 1, 1953. The nominations should be accompanied by such data relative to the nominee and his research as will facilitate consideration for the award.

Chairman, Nominating Committee:

DR. LEO T. SAMUELS
Department of Biological Chemistry
University of Utah Medical School
Salt Lake City, Utah